Deciphering the ecological functions of fungal root endophytes based on their natural occurrence

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List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
DSE	Dark Septate Endophytes
HTS	High-throughput sequencing
ITS	Internal Transcribed Spacer
OTU	Operational Taxonomic Unit

Summary

Plants are colonized by a large diversity of fungi, some residing on the surface and others penetrating the plant tissues, the latter referred to as fungal endophytes (*endon* Gr., within; *phyton*, plant; de Bary 1879). Despite the saprotrophic potential of fungal endophytes, they are not found to cause visible disease symptoms to the host. Plants are colonized simultaneously by various fungal species, which form rich and diverse endophytic assemblages. Although it is hypothesized that fungal endophytes contribute to the fitness of their hosts and to the functioning of ecosystems, the ecological function of fungal endophytic assemblages remains cryptic. The aims of this doctoral thesis are to gain insight to the ecological functions of root fungal endophytes, by deciphering their roles in ecosystems based on their natural occurrence and the structure of their assemblages. The thesis focuses on studying the diversity and structure of the endophytic mycobiome within roots of two annual and widespread plant hosts *Microthlapsi perfoliatum* and *M. erraticum* (Brassicaceae) in several locations across northern Mediterranean and central Europe. The thesis is composed by six Chapters, with a primary focus on Chapter 1, 2 and 3.

Chapter 1 (Glynou *et al*., 2016) aimed at characterizing the diversity of fungal endophytes in roots at a continental scale and at assessing the factors affecting the structure of endophytic assemblages with the use of cultivation-based methods. For that, root samples were collected from 52 plant populations, along with a collection of soil, bioclimatic, geographic and host data. Cultivation of surface-sterilized root samples on culture media and isolation of fungal colonies in pure culture generated 1,998 fungal colonies. Grouping of sequences into Operational Taxonomic Units (OTUs), based on the 97% similarity of the isolates' rDNA Internal Transcribed Spacer (ITS) sequence, generated in total 296 OTUs, representing taxa mostly within the phylum Ascomycota with a minor representation of Basidiomycota. Endophytic assemblages were mostly correlated with variation in bioclimatic conditions. Interestingly, despite the large diversity revealed, the assemblages were dominated by only six OTUs related to the orders Hypocreales, Pleosporales and Helotiales, which had a widespread distribution across populations but with some following patterns of ecological preferences.

Chapter 2 aimed at characterizing the uncultivable fraction of the root fungal endophytic diversity, which was not possible to capture in Chapter 1. High-throughput sequencing via the

Illumina Miseq platform was implemented in 43 of the 52 original populations and mostly in the same root samples. In comparison with the cultivation-based approach, the HTS managed to cover the overall diversity within samples. It revealed a large non-cultivated endophytic diversity but the same cultivable fungi dominated assemblages. Moreover, the endophytic diversity was grouped mostly within fungal orders with demonstrated ability to grow in culture and taxonomically related groups were found to have divergent ecological preferences.

The genetic identity of the most abundant OTUs was further investigated in Chapter 3 (Glynou *et al*., 2017), aiming to unravel genotypic variability, which was possibly overlooked due to the use of lTS, as a universal genetic marker, and could explain their high abundance and widespread distribution. Multi-locus gene sequencing and AFLP profiling for the five most abundant OTUs suggested a low within-OTU genetic variability and show that these fungi have ubiquitous distribution and are not limited by environmental conditions within the ecological ranges of the study. A selection of endophytes frequently isolated in Chapter 1 was functionally characterized in Chapter 4 (Kia *et al*., 2017) based on the isolates' traits and interactions with plants. In Chapter 5 (Cheikh-Ali *et al*., 2015) fungal cultures of *Exophiala* sp. with differential colony structure where investigated for their production of secondary metabolites. Moreover, Chapter 6 (Maciá-Vicente *et al*., 2016) comprises the description of the new species *Exophiala radicis* based on morphological and molecular characteristics.

Compilation of all results shows that the fungal endophytic diversity in roots of *Microthlaspi* spp. is high but few widespread OTUs dominate the assemblages, and have unlimited dispersal ability. These fungi seem also to have a wide niche breadth and are not affected by environmental filtering. The findings indicate that the local environment but also processes of competitive exclusion determine the structure of endophytic assemblages. In addition, the fungal endophytes associated with *Microthlapsi* spp. likely have saprotrophic activity however the interactions with plants are likely context-dependent. Further research is needed to assess the biotic interactions among endophytes and their effect on the structure of fungal endophytic assemblages. Ultimately, the findings of this thesis are useful to shed light on the processes underlying the structure of endophytic assemblages. They also upraise the need to describe diversity by combining genetic, metabolic and physiological data, in order to disentangle the elusive ecological roles of the endophytic mycobiome.

Zusammenfassung

Pflanzen sind von einer großen Vielfalt von Pilzen besiedelt, von denen einige auf der Oberfläche und andere im Pflanzeninneren leben. Die letzteren werden Endophyten genannt, basierend auf ihrer Fähigkeit, das Innere des pflanzlichen Gewebes zu besiedeln (*endon* Gr., innerhalb; *Phyton*, Pflanze; de Bary 1879). Endophyten stellen eine vielfältige und allgegenwärtige Gruppe von Pilzen dar. Trotz des saprotrophen und pathogenen Potenzials von Endophyten verursachen sie keine sichtbaren Krankheitssymptome an Pflanzen, jedoch ist ihre Interaktionen mit Pflanzen noch nicht richtig verstanden.

Eine große Vielfalt von endophytischen Pilzen wurde anhand kultivierungsbasierter Methoden dokumentiert. Bei diesen Pilzen handelt es sich meist um verschiedene Arten innerhalb der Abteilung Ascomycota. Basidiomycota sind unter den Endophyten seltener vertreten und werden häufig in holzigen Pflanzen gefunden. Es ist auch bekannt, dass die Vielfalt von endophytischen Pilzen zwischen verschiedenen Pflanzengeweben variiert und von deren Organanatomie und -chemie abhängt. Allerdings ist die Beschreibung der gesamten endophytischen Pilzvielfalt durch die Unkultivierbarkeit vieler Pilzarten eingeschränkt. Die Implementierung der Hochdurchsatz-Sequenzierung hat gezeigt, dass die Pilzvielfalt viel größer ist als bisher angenommen. Auch scheint es, dass das Mykobiom der Pflanzen eine Quelle unerforschter Vielfalt ist.

Es wurde gezeigt, dass viele verschiedene Pilzendophyten eine Pflanze gleichzeitig kolonisieren. Sie bilden artenreiche und vielfältige endophytische Gesellschaften, aber es ist noch unklar welche ökologischen Faktoren prägen die Zusammensetzung und auf welche Weise. Mehrere Studien zeigen, dass das pflanzenassoziierte Mykobiom von verschiedenen ökologischen Faktoren bestimmt wird, wie räumliche Variablen, Wirtsgenotyp und bioklimatische Gegebenheiten. Allerdings lassen neue Studien vermuten, dass auch stochastische Prozesse, die sich auf die Ausbreitungsbeschränkungen und die historische Kontingenz beziehen, die Artenzusammensetzung beeinflussen können. Diese Diskrepanz

wird durch die Schwierigkeit erhöht, das Verbreitungsgebiet von Pilzen zu definieren. Es wird vermutet, dass Pilze, die zur Gruppe der Mikroorganismen gehören, aufgrund ihrer mikroskopischen Größe und zahlreiche Sporen eine ubiquitäre Verbreitung erreichen und nur durch Filterungsprozesse in der Umwelt begrenzt sind. Doch jüngste Ergebnisse weisen auf eine hohe endemische Verbreitung von Mikroorganismen hin.

Wegen der großen Vielfalt an Pilzendophyten ist es schwierig, die Interaktionsmuster mit Pflanzen und ihre ökologischen Funktionen aufzuklären. Untersuchungen über die Auswirkungen einzelner Endophyten auf Pflanzen unter Stressbedingungen haben gezeigt, dass sie positive Wirkungen auf die Pflanzen haben und diese vor Umweltbelastung schützen. Mehrere Studien berichten jedoch über schädliche Wirkungen von Endophyten auf Pflanzen. Es wird daher vermutet, dass diese Interaktionen einem ausgeglichenen Antagonismus zwischen Endophyten und Pflanzen unterliegen, was bei Änderung der ökologischen Bedingungen zu negativen Auswirkungen auf Seite der Pflanzen oder der Endophyten führen kann. Darüber hinaus sind Pilzendophyten wahrscheinlich wichtig für das Ökosystem, da sie die Zusammensetzung der unter- und oberirdischen Mikrobiom bestimmen. Zusätzlich tragen sie wahrscheinlich eine entscheidende Rolle bei zum Nährstofftransport im Boden und entsprechend auch an der Ökosystemfunktion, jedoch sind die Zusammenhänge noch unklar.

Das Ziel dieser Dissertation ist einen Einblick in die ökologischen Funktionen von endophytischen Pilzen der Wurzel anhand ihres natürlichen Vorkommens und ihrer Artenzusammensetzung zu geben. Die Arbeit konzentriert sich auf das Studium der Vielfalt und der Struktur des endophytischen Mykobioms innerhalb der Wurzeln von den zwei einjährigen und weitverbreiteten Pflanzen *Microthlapsi perfoliatum* und *M. erraticum*. Die Wurzelproben wurden innerhalb einer Studie erhoben, welche im Frühjahr und Sommer 2013 im nördlichen Mittelmeerraum und Mitteleuropa durchgeführt wurde und sich über sieben Länder erstreckt. Die Pflanzen sind der Familie der Brassicaceae zugeordnet und gehörten früher beide zu der Art *M. perfoliatum*, vor kurzem wurden die Arten jedoch getrennt. Diese Pflanzenarten sind weit verbreitet, daher eignen sie sich sehr gut für ökologische Studien da sie ein breites Spektrum an ökologischen Bedingungen abdecken. Darüber hinaus sind diese Pflanzen gut dazu geeignet, Symbiosen mit Endophyten in Abwesenheit von mykorrhizalen Symbiosen zu untersuchen. In dieser Promotion wird ausschließlich auf das endophytische Mykobiom der Wurzeln eingegangen, denn obwohl die Wurzel ein lebenswichtiges Organ für die Ernährung von Pflanzen darstellt, ist das mit den Wurzeln assoziierte Mykobiom im Vergleich zum Blatt-Mykobiom wenig untersucht.

Die Arbeit besteht aus sechs Kapiteln mit einem Schwerpunkt auf Kapitel 1, 2 und 3.

Kapitel 1 (Glynou *et al*., 2016) zielte darauf ab, die Pilzvielfalt der Wurzelendophyten von verschiedenen Standorten zu charakterisieren und die Faktoren zu beurteilen, die die Struktur der endophytischen Artenzusammensetzung beeinflussen. Dafür wurden 3-10 Wurzelproben aus 52 Pflanzenpopulationen gesammelt. Bodenproben aus 42 Populationen wurden zur Analyse der physikalisch-chemischen Eigenschaften des Bodens untersucht. Darüber hinaus wurden Daten über die Geographie, die bioklimatischen Bedingungen und dem Pflanzengenotyp für jede Population aufgenommen und alle Variablen, einschließlich des Wirtsgenotyps, wurden als ökologische Faktoren für die Bewertung ihres Einflusses in der Struktur der endophytischen Artenzusammensetzung einbezogen. Die Isolierung der Pilz-endophytischen Diversität umfasste die Reinigung und die entsprechende Sterilisation der Wurzeloberflächen für den Ausschluss von Pilz-Epiphyten, die Kultivierung von Wurzelproben auf Kulturmedien und die Isolierung von Pilzkolonien in Reinkultur. Die Charakterisierung von Kulturen beruhte auf der genetischen Identifizierung durch DNA-Extraktion, Amplifikation und Sequenzierung des Internal Transcribed Spacers (ITS), eines universellen genetischen Barcodes für Pilze. Sequenzen wurden in Operational Taxonomic Units (OTUs) gruppiert, basierend auf der Ähnlichkeit ihrer ITS-Sequenz von 97%. Die Analyse der Vielfalt basierte auf der Einschätzung von Abundanz, Artenreichtum und Vielfalt und wurde mit der taxonomischen Beschreibung der generierten OTUs ergänzt. Die Analyse ergab, dass Pilz-endophytische Gesellschaften aus 296 OTUs zusammengesetzt sind, von der die meisten Arten in die Abteilung Ascomycota und nur ein kleiner Anteil in Basidiomycota eingeordnet werden konnten. Der endophytische Artenreichtum war negativ mit dem jährlichen Niederschlag korreliert. Darüber hinaus zeigte die Assoziation von Pflanzengenotypen, geographischen und umweltbezogenen Variablen mit der Zusammensetzung endophytischer Artengesellschaften, dass ihre Struktur von den lokalen Gegebenheiten und meist von bioklimatischen Bedingungen beeinflusst wird. Interessanterweise dominierten Artengesellschaften aus nur sechs OTUs, die zu den Ordnungen Hypocreales, Pleosporales und Helotiales gehörten. Die Gruppen hatten zwar eine weit verbreitete Verteilung über die Populationen, jedoch zeigten einige ökologische Präferenzen.

Kapitel 2 zielte darauf ab, den unkultivierbaren Anteil der Vielfalt von endophytischen Pilzen zu charakterisieren. Dafür wurde in 43 der untersuchten Populationen eine Hochdurchsatz-Sequenzierung meist an denselben Pflanzen durchgeführt. Die Sequenzierung über die Illumina Miseq-Plattform, die Sequenzverarbeitung und die OTU-Abgrenzung anhand

einer Ähnlichkeit von 97% führte zu insgesamt 995 OTUs. Im Vergleich zum kultivierungsbasierten Ansatz war die Hochdurchsatz-Sequenzierung geeignet, die Gesamtdiversität aufzudecken und produzierte eine viel größere Menge an OTUs. Allerdings zeigen die Ergebnisse die Dominanz der gleichen kultivierbaren OTUs mit weitverbreiteter Verteilung aber auch, dass viele seltene Arten lokal begrenzt sind. In einem Versuch die Gesellschaften funktional zu klassifizieren, wurde festgestellt, dass ein großer Teil der endophytischen Vielfalt zuvor durch Kultivierungsmethoden nachgewiesen wurde. Die taxonomischen Ergebnisse zeigten, dass die Mehrheit der OTUs zu Pilzen gehörten, die kultivierbar sind und auch, dass sie taxonomisch ähnlich zu OTUs aus Kapitel 1 sind. Die ökologische Analyse der Faktoren, die die Struktur der Artenzusammensetzung beeinflussen bestärkt das vorher erkannte Muster eines starken Einflusses von bioklimatischen Variablen. Die Wirkung von räumlichen und umweltbedingten Faktoren wurde auch an einzelnen OTUs untersucht und war nicht phylogenetisch konserviert. Das deutet darauf hin, dass endophytische Taxa, die phylogenetisch ähnlich sind, unterschiedliche Nischen besetzen.

Die genetische Identität der sechs am häufigsten vorkommenden OTUs wurde in Kapitel 3 weiter untersucht, um die genotypische Variabilität innerhalb der OTUs zu entschlüsseln, die durch den Einsatz von ITS ohne ausreichender genetischer Auflösung übersehen werden. Haplotyp Netzwerkanalysen für die ITS-Sequenzen in Kombination mit Multilocus Gen Sequenzierung und AFLP Profiling für die fünf häufigsten OTUs ergaben eine niedrige genetische Variabilität innerhalb der OTUs. Dies bestätigt die Ergebnisse aus Kapitel 1, bei der die Dominanz der endophytischen Gemeinschaften durch wenige Taxa gezeigt wurde. Darüber hinaus zeigen die Ergebnisse, dass die Pilze weit verbreitet sind und interessanterweise nicht durch Umweltbedingungen innerhalb der ökologischen Bereiche begrenzt sind. Allerdings gibt es Hinweise darauf, dass *Cadophora* sp. Isolate viel vielfältiger sind als vermutet und mehr Anstrengungen zur Charakterisierung dieses Taxons notwendig sind.

In Kapitel 4 (Kia *et al*., 2016) wurde eine Auswahl von Endophyten, die häufig in Kapitel 1 isoliert wurden, funktionell auf der Grundlage der Eigenschaften der Isolate und der Interaktion mit Pflanzen charakterisiert. *In vitro* Beurteilung zeigte, dass die meisten Endophyten ein parasitärer Effekt auf Pflanzen haben. In Kapitel 5 (Cheikh-Ali *et al*., 2015) wurde festgestellt, dass Pilzkulturen von *Exophiala* sp., welche für Kapitel 1 isoliert wurden, eine Differentialkoloniestruktur aufweisen. Die Untersuchung von Pilzkulturen auf

ihr metabolisches Profil zeigte, dass ihre Morphologie mit einer Variation der Struktur der Exophilsäure einhergeht. Darüber hinaus schließt Kapitel 6 (Maciá-Vicente *et al*., 2016) eine umfassende Bewertung der phylogenetischen Verwandtschaft der Isolate ein. Durch die Sequenzierung von zusätzlichen Genen in Kombination mit der mikromorphologischen Beschreibung wurde die neue Art *Exophiala radicis* beschrieben.

Diese Dissertation erweitert das bisherige Wissen über die Vielfalt der Pilzendophyten in den Wurzeln der Pflanze *Microthlaspi* spp. innerhalb Europas. Die Zusammenstellung aller Ergebnisse zeigt, dass die Vielfalt zwar hoch ist, aber die dominierenden endophytischen Gruppen über alle untersuchten Pflanzenpopulationen gleich sind. Ihre Ubiquität der Pilze in Kombination mit ihrer niedrigen genotypischen Variation kann durch die Tatsache erklärt werden, dass sie unbegrenzte Ausbreitungsfähigkeit haben. Interessanterweise scheinen diese Pilze auch eine breite Nische zu bewohnen, und nicht von der spezifischen Variation der Umgebungsbedingungen betroffen sind. Die Zusammensetzung der Pilz-endophytischen Gesellschaft wird von der lokalen Umgebung beeinflusst, vermutlich mit stärkere Wirkung auf die Etablierung von seltenen Pilzen als auf diejenige von dominanten Pilzarten. Da allerdings kein phylogenetischer Konservatismus auf die Verbreitung von Pilzendophyten gefunden wurde, scheint es, dass die Variation in der Artenzusammensetzung ein Effekt von Nischenpartitionierung sein könnte. Das wurde auch für andere Pilze gefunden, und ist ein Mechanismus, um den Wettbewerb um dieselbe Ressource zwischen Pilzen mit ähnlichen Merkmalen zu verringern. Darüber hinaus sind die häufigen Taxa nicht so stark von Umweltbedingungen beeinflusst, was stattdessen vermuten lässt, dass es eine wettbewerbsbedingte Verdrängung und Prioritätseffekte gibt. Solche Prozesse begünstigen die frühen Kolonisatoren und können den signifikanten Effekt von räumlichen Variablen in der Struktur des endophytischen Mykobioms erklären.

Die Kultivierbarkeit der Mehrheit von Pilzendophyten sowie die Ergebnisse des *in-vitro*-Experiments auf die Pflanzen-Pilz-Wechselwirkung zeigen, dass die Pilzendophyten in *Microthlapsi* spp. saprotrophische Aktivität haben und dass diese Pflanzen keine Assoziation mit biotrophen Pilzen entwickeln. Pflanzenwurzeln werden möglicherweise von Endophyten als Ressource von Kohlenstoff und anderen Nährstoffen kolonisiert. Die Ergebnisse zeigen was schon vorgeschlagen, dass Endophyten wahrscheinlich neutral bleiben, solange Pflanzenwurzeln lebenswichtig sind. Nach Absterben der Wurzel werden sie zu Saprobionten um die Ressource weiter nutzen zu können. Allerdings können Endophyten möglicherweise auf die Besiedlung der Wurzel als Mittel zur Vermeidung von Konkurrenz innerhalb des

reichen Bodenmikrobioms zurückgreifen. In diesem Fall kann die hohe Fülle von wenigen Pilz-Taxa in Wurzeln von *Microthlaspi* spp. auf ihre starke Anpassung an die typische Wurzelumgebung und ihre Dominanz gegenüber anderer endophytischer Taxa hinweisen. Weitere Studien sollten den Grad der aktiven Rekrutierung von Endophyten durch den Wirt untersuchen, aber auch die Wirkung der biotischen Wechselwirkungen zwischen den Endophyten in der Struktur von Pilz-endophytischen Artengesellschaften. Letztendlich stellen die Ergebnisse dieser Arbeit nützliche Hypothesen dar, um die zugrunde liegenden Prozesse zu Mustern der endophytischen Artenzusammensetzung zu identifizieren. Diese Arbeit zeigt auch die Notwendigkeit der Beschreibung von Vielfalt, indem genetische, metabolische und physiologische Daten kombiniert werden müssen, um die schwer fassbaren ökologischen Rollen des endophytischen Mykobioms zu entwirren.

General Introduction

1. Fungal endophytes

Plants serve as hosts for numerous microorganisms that are able to colonize the outer plant parts or penetrate the plant tissues (Porras-Alfaro and Bayman, 2011). Many microscopic fungi are found colonizing asymptomatically the interior of plants without causing visible disease symptoms, which are referred to as fungal endophytes (*endon* Gr., within; *phyton*, plant; de Bary 1879; Schulz & Boyle 2005; Rodriguez *et al.* 2009). Fungal endophytes are widespread in ecosystems and form diverse assemblages within plant tissues (Porras-Alfaro and Bayman, 2011). The majority of fungi that have been found to adopt an endophytic lifestyle in roots or foliage belong to the phylum Ascomycota (Schulz and Boyle, 2005), with a smaller representation of Basidiomycota, which are more frequent in woody tissues (Rodriguez *et al.*, 2009). While endophytes can colonize various plant organs, the endophytic diversity is to a certain degree organ-specific (Arnold, 2007). This distinction is due to the physicochemical and anatomical differences in the plant compartments, which subsequently determine the microbiota inhabiting these tissues as well as the patterns of colonization by microorganisms (Schulz and Boyle, 2005). Endophytes interact with their hosts and are considered to have important effects on plant performance. An example is the Clavicipitaceae endophytes within the genus *Epichloë*, which develop symbiotic interactions with plant species of the family Poaeae. The relationship of these endophytes with their host is an example of coevolving interactions linked to defensive mutualism (Saikkonen *et al.*, 2016). The *Epichloë* endophytes protect grasses via their production of secondary metabolites and induction of toxicosis in herbivores in exchange of nutrients by their hosts (Faeth and Fagan, 2002; Kuldau and Bacon, 2008; Rodriguez *et al.*, 2009). However, for the majority of fungal endophytes within plants, their effect on plant fitness and ecosystem function still remain cryptic (Mandyam and Jumpponen, 2005; Glynou *et al.*, 2016).

The endophytic mycobiome has attracted scientific attention due to the general interest to explore the microbial diversity and understand the ecological importance of microbes in ecosystems, but also due to the functional implication of their interactions with plants, which remains elusive (Vandenkoornhuyse *et al.*, 2015). In addition, despite the large fungal diversity already reported, global estimations predict that the diversity is larger than currently considered (Taylor *et al*., 2014). In fact, the plant-associated mycobiome is considered a source of hidden diversity, which remains to be explored (Blackwell, 2011; Peay *et al.*, 2016). It is also a pool of fungal species with great potential in the production of new antibiotics in the era of emerging antibiotic resistant pathogens, owing to the metabolically active environment they are subject to (Kusari *et al.*, 2012).

1.1. Fungal endophytes in plant roots

Plant roots, due to their contribution to plant nutrition, have developed important symbiotic interactions with different groups of mycorrhizal fungi that assist in the transfer of phosphorus and other nutrients in exchange for photosynthetic carbon (Smith and Read, 2007). Apart from the mycorrhizal fungi and their distinctive symbiotic roles, fungal endophytes also colonize extensively root tissues both inter- and intra-cellularly. In comparison with mycorrhizae, fungal endophytes do not form characteristic infective structures (Saikkonen *et al*. 1998) and their interactions with the host are still not clearly defined (Brundrett, 2006; Schulz and Boyle, 2005). However, the colonization of roots by endophytic fungi is considered to be as old as the mycorrhizal symbioses, therefore endophytic symbioses are likely to be involved in important evolutionary processes of plants (Andrade-Linares and Franken, 2013). Fungal root endophytes comprise a large diversity within various taxonomic groups. They involve fungal species that can infect plants endophytically but which can be also free-living in the soil. Such fungi are mostly members of the Helotiales, Pleosporales, Xylariales, Hypocrelaes within Ascomycota, and Cantharellales and Sebacinales within Basidiomcota (Andrade-Linares and Franken, 2013; Schulz and Boyle, 2005). Among the best-studied group is that of the dark septate endophytes (DSE), which are widespread in most plants (Andrade-Linares and Franken, 2013). This group comprises fungi within various lineages named after their melanized hyphae (Arnold,

2007). While the functional roles of DSE are still under investigation, they are considered to help plants tolerate abiotic stress, involving nutrient transfer and assistance in water uptake (Mandyam and Jumpponen, 2005; Newsham, 2011).

In general, the root-associated microbiome is considered to be different from that colonizing the rest of the plant, as it is determined by the soil type and the host growing stage, whereas in the phyllosphere the diversity is more dependent on random dispersal of microbes through the air and the substrate chemistry (Lebeis, 2015). The patterns of microbial diversity seem also to vary, with the root microbiome being more stable among samples but locally more diverse, in comparison to leaves, where spatial diversity is less but more variable (Lebeis, 2015). However, other studies suggest that the compartmentalized plant canopies allow for restricted and hence more diverse microbial colonization, while we see a more systemic and less diverse microbiome in roots (Schulz & Boyle 2005; Rodriguez *et al.* 2009).

Among all plant tissues, roots were selected for exploration of the endophytic mycobiome, a tissue that still remains less explored than the phyllosphere, where the foliar endophytic mycobiome has been extensively studied (Arnold, 2007; Arnold *et al.*, 2007; Arnold and Lutzoni, 2007; Bálint *et al.*, 2015, 2013; Zimmerman and Vitousek, 2012). This is because roots constitute a plant organ important in the transport of nutrients and vital for the survival of the plant. Therefore, studying the endophytic diversity within roots may reveal plant-endophyte interactions with ecological significance. In addition, since roots are in contact with soil, which constitutes a diverse and well-studied pool of microbial species, the identification of the endophytic mycobiome could give us important information on the degree of selected recruitment by the host and the processes involved in endophytic colonization.

1.2. Selection of host for studying root fungal endophytes

This thesis focuses on the diversity of fungal root endophytes within only one plant genus along a latitudinal gradient which involves significant differences among spatial, soil and bioclimatic conditions. Initially, the plant selected was *Microthlaspi perfoliatum* (L.) F.K. Meyer, which comprised diploid and polyploid genotypes. Recent data, however, support that due to their phylogenetic dissimilarity, they represent two different species, named as *Microthlapsi erraticum* corresponding to the diploid genotype, and *Microthlaspi perfoliatum* corresponding to the polyploid genotype (Ali *et al*., 2015). The genus *Microthlaspi* belongs to the family of Brassicaceae and has some characteristics which makes it a good model for this study. *Microthlaspi* lacks mycorrhizal symbiosis, which is associated with their habitat preferences (Fitter, 2005). Therefore, the absence of mycorrhizae could imply presence of other endophytes, which may develop symbiotic interactions with their host and have ecological importance. In addition, they are annual plants, thus it makes them ideal to eliminate the effect of temporal variation in shaping communities, when interested solely for spatial variables. The genus *Microthlaspi* has a widespread distribution across Europe (Meyer, 2003), therefore it is ideal for biogeographic studies along a wide geographic range. Finally, this genus is closely related to the model plant *Arabidopsis thaliana*, and gives the opportunity for comparisons with existing data and use for physiological experiments to a well-established model but also genetically related to the original host species.

Focusing on two closely related plant species is an experimental set-up that has not been applied over long distances and large-scale samplings for the study of the endophytic mycobiome. So far, similar studies on endophytes have been conducted by comparing diversity in different environments, but in several host plants (Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; Herrera *et al*., 2013; U'Ren *et al*., 2012), which can make it difficult to distinguish the effect of individual factors from the total variation. Here, studying the same host genus for all sites, minimizes the effect of host phylogeny and allows for more accuracy in comparing results from different sampling sites. Nevertheless, since there is some degree of genetic variability of the hosts, data on the genetic profile of the sampled plant populations were collected and used to assess potential effect on the endophytic diversity. In addition, data on the geographic location, bioclimatic variables as well as soil physicochemical characteristics were collected to be used for the assessment of their contribution to the composition of endophytic communities.

2. Methods for studying the fungal endophytic diversity

The research on fungal endophytic diversity has been for long time based on methods of fungal cultivation on nutrient media, involving sampling of plants, cultivation of plant tissues in medium and isolation of growing fungal colonies. Sterilization of the surface of plant tissues is necessary to remove epiphytic fungi and ensure the exclusive isolation of endophytic fungi (Maciá-Vicente *et al.*, 2008, 2012; Porras-Alfaro and Bayman, 2011). So far, cultivation methods have been applied in various studies (Arnold *et al.*, 2007; Arnold and Lutzoni, 2007; Maciá-Vicente *et al.*, 2008, 2012; Keim *et al.*, 2014; Siddique and Unterseher, 2016), revealing a large endophytic fungal diversity associated with plants. However, these methods have certain limitations that can lead to underestimations of the total diversity, thus limiting the accuracy of the data (Peay *et al.*, 2008). Cultivation methods are not sufficient enough to cover hyperdiverse communities like those composed by endophytes, leading often to lack of characterization of the total diversity (Zimmerman *et al.*, 2014). In addition, there is large variability in culturability across fungal lineages, in that certain fungi can grow faster in culture and inhibit the development of slow-growing fungi. Moreover, there is a big number of fungal species that cannot be cultivated on media and therefore they are neglected when cultivation-based methods are used (Epstein, 2013; Zimmerman *et al.*, 2014). This can cause biases in the estimations of biodiversity and result in overestimation of the abundance of particular fungal groups (Porras-Alfaro and Bayman, 2011). In addition, the isolation of saprotrophic fungi can cause vague interpretations of the ecological roles of fungal endophytes, as these methods overlook the presence of obligate biotrophic taxa, since they are unable to grow in culture.

The use of high-throughput sequencing (HTS) methods has facilitated the research on microbial diversity, by overcoming the above limitations (Peay *et al.*, 2008). These methods are independent of cultivation and involve sequencing of the fungal diversity directly from the plant material after DNA extraction (Bálint *et al.*, 2016; Peay *et al.*, 2008; Zimmerman *et al.*, 2014). This approach provides identification of the non-cultivable diversity as well as slow-growing fungal taxa, uncovering a much larger diversity than cultivation-based methods (Degnan and Ochman, 2012; Schmidt *et al.*, 2013; Wehner *et al.*, 2014; Zimmerman *et al.*, 2014; Bálint *et al.*, 2015; Eusemann *et al.*, 2016). However, there are certain limitations with the application of HTS that need to be taken into account for the interpretation of the results it provides. Sequence heterogeneity and variability in amplification rates of genetic loci among

different species cannot be easily corrected during sequence analysis and can lead to quantification biases and over-representation of specific taxa (Medinger *et al.*, 2010; Lindahl *et al.*, 2013; Nguyen *et al.*, 2015). Porras-Alfaro and Bayman (2011) suggest a combination of both cultivation and sequencing methods to be the most comprehensive approach. Utilizing both methods is also be suitable to distinguish the fraction of cultivable and non-cultivable diversity. This would allow for some preliminary assessment of the ability of fungal endophytes to grow on culture media, which could be linked to their trophic lifestyles and the potential functions they serve to the host.

The taxa identification is another important element of the diversity analysis. When cultivation-based approaches are followed, the identification is possible either based on morphological characteristics of the fungal cultures, or based on sequencing of genetic loci or "barcodes". The microscopic characterization is essential when aimed to reach the species level (Taylor *et al.*, 2000) and is often based on the investigation of the reproductive structures. However, in the case of cultures deriving from fungal endophytes, the mycelium often remains sterile and lacks structures, thus morphological characterization of fungal endophytes is often not possible. In addition, the morphological identification of a large collection of cultures, representing very diverse groups, such as endophytes, is often time-inefficient. For these reasons, in studies of microbial diversity, the molecular identification is usually preferred and is based on a genetic locus appropriate for the respective organism. For fungi, there are several loci commonly used, the suitability of which can vary among taxa. The Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA is currently used as a universal barcode which facilitates the process of identification at a higher taxonomic level but also allows for comparisons among different studies (Schoch *et al.*, 2012). Among the advantages of using the ITS region in comparison to other loci are that it is easy to amplify in most fungi, gives a good estimation of the genetic variability and has a good representation in publicly available databases of DNA sequence data (Gazis *et al.*, 2011). Studies of fungi using HTS usually target the ITS region (Taylor *et al.*, 2014; Tedersoo *et al.*, 2014; Siddique and Unterseher, 2016; Bálint *et al.*, 2016; Eusemann *et al.*, 2016). The taxa delimitation is done with the generation of Operational Taxonomic Units (OTUs), which relies on the grouping of DNA sequences based on their sequence similarity with acceptable similarity thresholds between 95% and 99%. OTU delimitation does not constitute a formal taxonomic classification, but it is widely used in microbial diversity as it succeeds to delimit taxa in higher taxonomic groups and sometimes even to a near species level (Peay *et al.*, 2016).

However, the ITS region alone is not considered to be a sensitive marker with sufficient resolution and fails to fine-scale genetic variation among fungi (Nilsson *et al.*, 2008). For that reason, when aiming to unravel genetic differences within OTUs, methods with more genetic resolution are necessary (Taylor *et al.*, 2006; Gazis *et al.*, 2011). The implementation of multilocus gene sequencing, by sequencing additional genetic loci as well as genetic profiling, by screening the total genome with specific markers increases considerably the amount of information and thus the accuracy in assessing the phylogenetic relatedness of taxa (Mueller and Wolfenbarger, 1999; Taylor and Fisher, 2003). In addition, recent methods, such as whole-genome sequencing and high-throughput profiling, can provide more genetic information in a timely manner (Ellison *et al.*, 2011; Sperschneider *et al.*, 2015; Branco *et al.*, 2015; Wyss *et al.*, 2016).

3. Distribution patterns of fungal endophytes

Among the first steps to understand the ecology of microbes is the assessment of their patterns of distribution. Research on the mycobiome in roots has shown that their spatial structure is determined significantly by abiotic factors, which affect the establishment of fungal endophytes in space. Firstly, specific environmental conditions, such as low moisture or high salinity may act as an environmental filter and favor the establishment of species adapted to these conditions over other fungi (Peay *et al.*, 2016). On the other hand, stochastic processes also affect the distribution of fungi over long distances. Such processes are related to the dispersal ability of a fungus, which is determined by traits related to the organisms' dispersal, but also historical contigency, which refers to the fluctuations in species abundance due to historical events (Bahram *et al.*, 2016; Evans *et al.*, 2016). These processes, coupled with geographic features that cause spatial isolation like mountain ranges or water masses, can restrict the movement of fungi and hence influence their distribution (Agler *et al.* 2016; Peay *et al.* 2010; 2016).

Due to the many ecological variables that can potentially determine the microbial distribution, it is still under debate what forces are important in defining the ecological niche of microbes (van der Gast, 2015), including fungal endophytes. The small size of microbes and large number of their propagules indicates that they are able to maintain large population

sizes and have sufficient dispersal through the wind or water, succeeding to distribute ubiquitously over long distances (Finlay, 2002). Therefore, in contrast to the clearly defined ecological boundaries in the distribution of macroorganisms, microbes are thought to occur ubiquitously, and only environmental filtering restricts their distribution. This view is frequently stated with the so-called Baas-Becking hypothesis of 'everything is everywhere, but, the environment selects' (Baas-Becking, 1934). This hypothesis has been supported by several studies on the continental or global scale distribution of fungi (Queloz *et al.*, 2011; Davison *et al.*, 2015; Cox *et al.*, 2016). However other studies challenge this view, supporting that there is a certain degree of microbial endemicity, with microbes experiencing dispersal limitations and following distinct biogeographical patterns (Taylor *et al.*, 2006; Peay *et al.*, 2010a; Salgado-Salazar *et al.*, 2013; Ryšánek *et al.*, 2015).

The difficulty to clearly define geographical ranges in the distribution of fungi is magnified by the lack of accurate species recognition, which can lead to description of genetically divergent lineages as similar and overestimate their ubiquity (Taylor *et al.*, 2006). Species identification based on morphological description or on sequencing with universal genetic markers can underestimate the total fungal diversity (Peay *et al.*, 2008). Indeed, the implementation of HTS or the reliance on genetic markers more efficient to resolve taxa suggest that the degree of endemicity in fungi is much larger than what universal genetic markers show (Taylor *et al.*, 2006). This is because often there are cryptic species or intraspecific variation among fungi that remain undetected (Bickford *et al.*, 2007; Gazis *et al.*, 2011). Thus, increasing the genetic resolution of genetic markers for species delimitation is of great significance in fungi, and several studies show that intraspecific variation is associated with distinct biogeographic patterns. For example, Salgado-Salazar and colleagues (2013) investigated the genetic diversity among isolates of the cosmopolitan fungal species *Thelonectria discophora*. They reported 16 genetically divergent lineages and found that there is cryptic speciation and restricted geographical distribution among genotypes. Geml and colleagues (2008) investigated the global phylogenetic distribution of the generalist fungus *Amanita muscaria*, which can disperse through the wind. With the use of multilocus gene sequencing, they revealed a distinct phylogeographic structure for divergent lineages at a both intercontinental and intracontinental scale. Such intercontinental genetic divergence has been also reported for several other fungal species, collectively presented by Taylor and colleagues (2006). Importantly, the distribution of fungi can also have ecological implications, considering their contribution to several ecosystem processes (Peay *et al.*, 2010a). In fungal

endophytic symbioses, there is evidence of certain specificity of mycorrhizal genotypes toward host plants, which can influence their distribution range (Croll *et al.*, 2008). A similar pattern has also been reported for ubiquitous fungal pathogens. For example, the *Fusarium oxysporum* species complex comprises a large number of *formae speciales*, which are used to classify genetically distinct groups with the same species (Michielse and Rep, 2009). These groups are adapted to infect specific hosts and therefore follow the distribution of their hosts.

Identifying the patterns of distribution of fungal endophytes as well assessing the factors determining their range is an important step to understand their functional roles in relation to their host plants, and hence their overall ecological significance in ecosystems. For that, the implementation of methods with sufficient genetic resolution can significantly contribute to resolving the confusion of species delimitation and niche occupancy.

4. Fungal endophytic assemblages

After determining the distribution of endophytes, it is important to address how endophytes are organized in assemblages within host tissues. The research on endophytic assemblages focuses on identifying which species manage to colonize and establish within roots, what processes determine the organization of endophytic assemblages and what is their ecological impact. Large-scale studies, among different environments and hosts have been already conducted to study various fungal groups and have proven to be useful to elucidate which ecological factors determine the structure of fungal assemblages. For example, at a global scale, Tedersoo and colleagues (2014) found that climatic factors significantly affect the diversity of fungi in soil. Zimmerman and Vitousek (2012) studied the diversity of foliar endophytes along a broad elevation range and concluded on a strong effect of temperature and precipitation in structuring communities. On the other hand, Bálint and colleagues (2013) revealed a significant effect of host genotype in foliar mycobiome.

For root-associated fungi it is reasonable to expect an effect of soil properties in structuring assemblages, since roots are involved in transfer of nutrients from soil. In the case of mycorrhizal fungi, the nutrient availability, especially of phosphorus and nitrogen, are key factors in determining assemblages (Smith and Read, 2007). For fungal endophytes, Maciá-

Vicente and colleagues (2012) showed that at a local scale fungal assemblages in roots change significantly under the exposure to high soil salinity. However, despite the accumulating knowledge on the factors affecting the soil and foliage endophytic mycobiome, the studies on root endophytic assemblages are limited. Large-scale samplings are necessary and will allow to better resolve the ecological variables determining their structure.

4.1. Composition of fungal endophytic assemblages

The characterization of microbial assemblages relies on the assessment of the diversity and abundance of the taxa they contain. Studies on the fungal endophytic assemblages show a general pattern of few highly abundant fungal species dominating assemblages followed by a larger number of rare species (Zak & Willig, 2004), and reveal a dominance of Ascomycota over the Basidiomycota and other fungal phyla (Arnold *et al.*, 2007; Maciá-Vicente *et al.*, 2008; Keim *et al.*, 2014). However, the processes underlying the observed patterns are not yet clear. Peay and colleagues (2016) suggest that at a certain place the ecological preferences of fungi as well as their growth performance under the specific environmental conditions affect the structure of endophytic assemblages. The difference in the performance of fungi in the specific environment acts as a filter in either facilitating or hindering the establishment of species in the assemblages (Figure 1). But when the environmental pressure is low, the assemblage is more influenced by stochastic processes, which determine the order of arrival in a place. Consequently, these processes contribute to the structure of assemblages by introducing priority effects in favor of the earlier colonizers rather than the last (Figure 1). Such an effect has been experimentally shown in wood decomposer communities, where the immigration history caused differences in the final community assembly (Fukami *et al*. 2010).

Despite the importance of abiotic effects, biotic interactions are also crucial in defining the composition of microbial assemblages. Competition or facilitation between fungal lineages can either limit or favor their colonization of roots, which is also determined by the interaction with their host. For example, there is evidence that some species act as keystone species, which are important in sorting microbial colonization because their presence is interconnected with the presence of other taxa. Agler and colleagues (2016), by investigating bacterial and fungal assemblages of *Arabidopsis thaliana* and their co-

occurrence patterns, found that among a large microbial diversity, the presence of few species acted as stabilizers and affected the composition of the microbiome. Moreover, studies on leaf-associated mycobiome give evidence of indirect biotic effects, where primary colonization of leaves by pathogenic species can either suppress or activate the defense system of hosts, as well as change the host's metabolism (Agler *et al.*, 2016). This is induced by secretion of effector proteins (Mukhtar *et al.*, 2011) and can favor or hinder the later colonization by other fungal species.

Studying the phylogenetic structure of the endophytic mycobiome and the evolutionary relatedness of its fungal members can shed light on the processes underlying its structure. Interestingly, the phylogenetic relations among fungi could hint on whether biotic or abiotic processes are more important in sorting communities (Vamosi *et al.*, 2009). For example, as Cavender-Bares and colleagues (2009) state, since phylogenetically related species often share traits, it is expected that under the effect of environmental preference, assemblages contain phylogenetically related species. However, if biotic interactions are more important, then under strong competition for the same resource the communities should be constituted by species phylogenetically divergent, which share different functional traits and complement each other. To this end, the assessment of the phylogenetic relatedness of taxa can be used as a proxy to define the amount of shared traits, as well as to evaluate the magnitude of niche partitioning underlying the structure of assemblages (Srivastava *et al.*, 2012). Based on knowledge on the phylogenetic relations among members of highly diverse fungal soil assemblages, Taylor and colleagues (2014) observed a tendency of genetically related species to occupy divergent niches. In the case of endophytic assemblages, this concept could explain differential niche occupancy between soil and rhizospheric environments. It would also suggest that certain fungi under strong competition are forced to colonize new niches, such as roots, to escape competition with soil microbiota. However, whether there is evidence of such a pattern of distribution for fungal root endophytes is still not clear (Kia *et al* 2016).

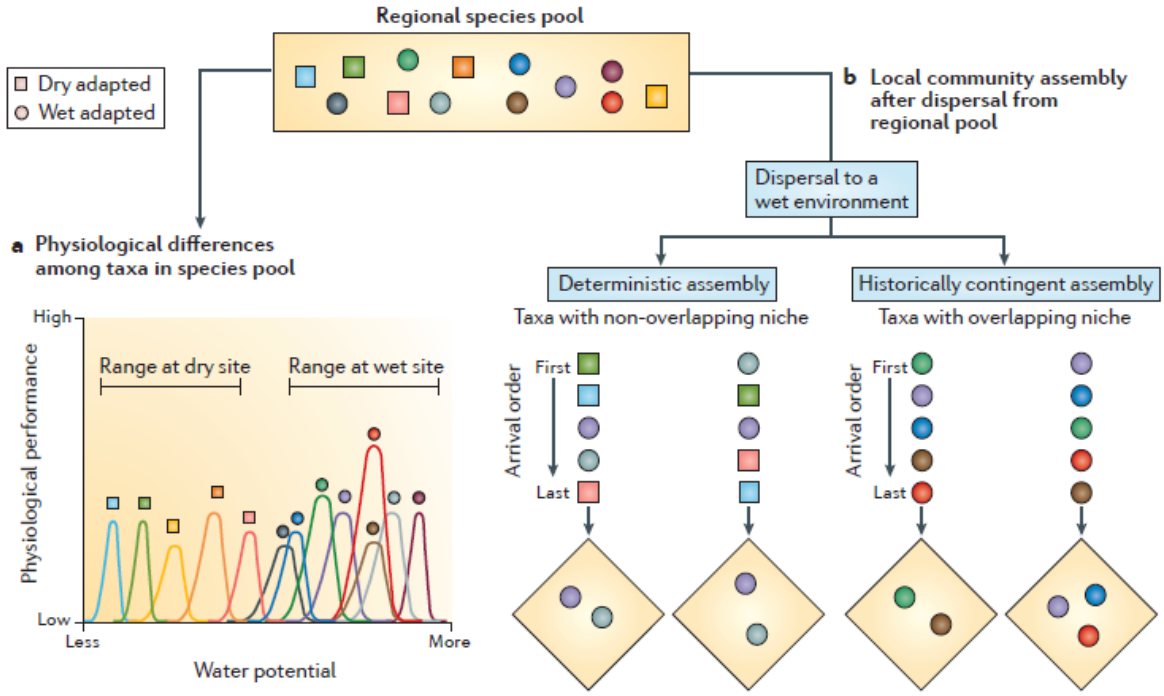

Figure 2. Deterministic and stochastic processes affecting the composition of fungal assemblages. When taxa with different environmental preferences arrive in a specific place, under the effect of environmental pressure, the taxa able to withstand the specific environmental conditions will thrive (a). But, among taxa with preferences for the same environmental conditions (niche overlapping), then the composition of assemblages is under the effect of stochastic factors, such as historical contingency, which define the order of arrival (b). Taken from Peay and colleagues (2016) with permission of Springer Nature.

From the above, it is conceivable that apart from the clear effect of deterministic and stochastic processes, microbe-host and microbe-microbe interactions seem to be equally important contributors to the composition of assemblages. However, considering that the endophytic mycobiome constitutes a complex environment, with multiple factors determining its structure, it is crucial to disentangle the magnitude of their effect. This will be necessary in order to elucidate the ecological processes involved in the establishment of host-microbe symbioses and the roles of fungal endophytes on plant performance.

5. Ecological significance of fungal endophytes

The characterization of the fungal endophytic diversity and the ecological processes determining the structure of fungal endophytic assemblages are necessary to assess the ecological significance of fungal endophytes for the host and the ecosystems. Several studies have revealed positive effects of individual endophytes on plant growth, which are associated with the secretion of plant hormones, the activation of metabolic pathways (Rodriguez *et al.*, 2009) and the production of secondary metabolites (Hardoim *et al.*, 2015). There is evidence that with the establishment of mutualistic interactions, the endophytes protect their hosts against plant pathogens and herbivores, such as the clavicipitateous endophytes (Rodriguez *et al*. 2008), but they are also thought to help plants to tolerate hostile environmental conditions. For example, the fungus *Curvularia protuberata* and its host manage to withstand high temperatures only via their symbiotic interactions (Márquez *et al*. 2007). Thermotolerance has been also reported for *Colletotrichum* spp. (Redman *et al.*, 2002), whereas symbiosis of plants with *Fusarium culmorum* has been found to contribute to the survival of both symbionts under high salinity (Rodriguez *et al.*, 2008). It has been suggested that fungal endophytes also contribute to nutrient transfer, as for example the DSE which may be involved in the utilization of organic nitrogen (N) from soil and contribute to the nutrient uptake by the host (Mandyam and Jumpponen, 2005). On the other hand, fungi are thought to tolerate environmental stress, possibly through the colonization of plant tissues, the interchange of nutrients and consequently the improvement of their nutrition status (Porras-Alfaro *et al.*, 2011). However, the pathogenic and saprotrophic characteristics of several endophytic groups makes it difficult to identify how they manage to contribute positively to the performance of their host (Kia *et al.*, 2017). For example, the beneficial effect against pathogens may also be due to the antagonistic interactions among endophytes within the same community (Porras-Alfaro *et al.*, 2011), which indirectly protect the host from invasions by triggering the host defense system prior to the infection by pathogens. In addition, despite reported positive effects of specific endophytic species on plant performance, there are also reported pathogenic effects of fungi against the host (Tellenbach *et al.*, 2011).

Considering the opposing findings on the plant-endophyte symbiosis, it is suggested that the interactions are subject to a balanced antagonism between fungal pathogenicity and the host's defense system (Schulz and Boyle, 2005). This antagonism, however, varies from mutualistic to antagonistic interactions and consequently determines the outcome of plant-

endophyte interactions (Schulz and Boyle, 2005). In asymptomatic host tissues the balance is maintained, but environmental changes which influence the host defense system may trigger the transition of an endophytic interaction into a pathogen (Porras-Alfaro and Bayman, 2011). Porras-Alfaro and Bayman (2011) also discuss the transition of endophytes to saprotrophs after host plant senescence, which is supported by the phylogenetic relatedness of endophytes with saprotrophic species (Figure 2). Considering nevertheless the large diversity of fungal endophytes, it is challenging to assess how the whole endophytic mycobiome determines the interaction with the host and what is its effect plant performance. It is also necessary to identify those individuals within the large endophytic diversity in roots that determine most of the changes on the interactions and affect the host performance.

Fungal endophytes are also considered to have important ecological roles on the ecosystems, since in addition to their direct effects on plant growth they can also indirectly affect the below and above-ground microbial communities and contribute to multitrophic interactions (Rudgers & Clay, 2005). For example, the colonization of roots by endophytes may be beneficial to suppress the expansion of soil microbes, pathogenic to plants. Nevertheless, saprotrophic fungi are significant contributors to the carbon cycle in the soil by decomposing plant litter and moving carbon through the soil (Blackwell, 2011; Hawkes *et al.*, 2011; Tedersoo *et al.*, 2014), whereas mycorrhizal fungi are able to incorporate soil carbon and are involved in transfer of nutrients (Hawkes *et al.*, 2011). It is therefore likely that fungal endophytes may be involved in nutrient transfer and have important implication on ecosystem functioning (Rudgers *et al.*, 2004). Understanding how endophytic communities function may therefore serve as a useful tool in crop management, but also in controlling emerging ecological phenomena such as climate change (Porras-Alfaro and Bayman, 2011).

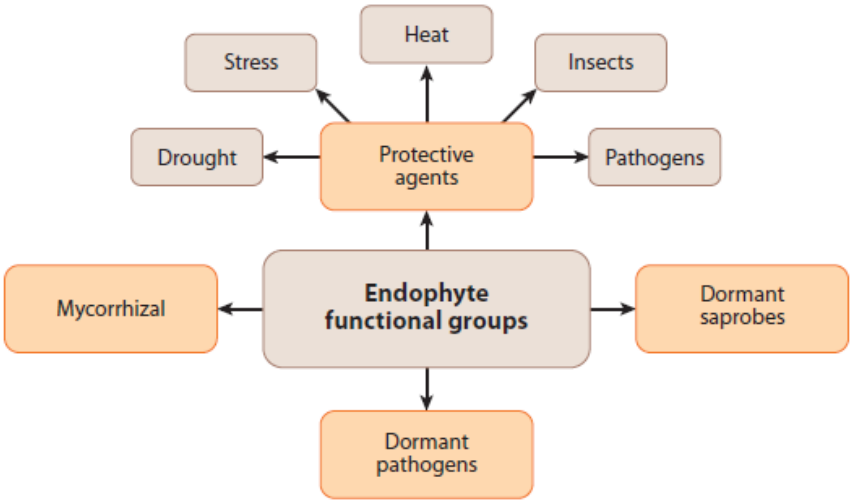

Figure 2. Suggested functions of fungal endophytes. Under varying conditions endophytes can change roles, from beneficial agents, such as mycorrhizal fungi against environmental stress and nutritional deficiency, to pathogenicity and saprotrophism. The factors triggering endophytic pathogenicity are related to environmental conditions as well as to biotic interactions. Fungal endophytes can also act as pathogens or saprobes in dormancy, which are activated after plant senescence. Taken from Porras-Alfaro and Bayman (2011) with permission of Annual Reviews.

6. Structure and aims of the doctoral thesis

The main objective of this thesis is to shed light on the ecological functions of fungal root endophytes based on their patterns of occurrence and the structure of their natural assemblages. In order to achieve this objective, the following specific aims will be addressed:

1. describing the diversity of fungal endophytes in roots of *Microthlaspi* spp.
2. assessing the distribution of fungal endophytes and their ecological preferences
3. studying the composition of endophytic assemblages and assess the ecological factors determining their structure
4. and combining physiological, metabolic and genetic data as an integrated approach to study the ecology of root fungal endophytes

This thesis is structured in six Chapters. My main contribution corresponds to Chapters 1–3, which focus on tackling different objectives 1–3. Chapters 4–6 jointly address the fourth aim. Below, a brief outline of the content of each chapter is provided. Chapter 1 (Glynou et al., 2016) is aimed at exploring the diversity of fungal endophytes within roots of *Microthlaspi* spp. in different localities using cultivation-based approaches. It also aimed at assessing the composition of the endophytic mycobiome and the ecological factors determining its structure and generating a collection of isolates for further experiments. In Chapter 2 the diversity of fungal endophytes is studied with the use of high-throughput sequencing. It compiles a comparison between cultivation and non-cultivation based methods, and aims at classifying the endophytic diversity functionally based on their ability to grow in culture. Chapter 3 (Glynou *et al*., 2017) focused on assessing possible intra-group genetic variability among the dominant fungal groups of Chapter 1. It also addressed what ecological principles apply in the distribution of root fungal endophytes. Chapter 4 (Kia *et al*., 2017) focuses on a fraction of the root fungal endophytic diversity isolated in Chapter 1 and aimed at assessing the effects of root fungal endophytes on plant performance. Chapter 5 (Cheikh-Ali *et al*., 2015) includes the description of metabolic compounds produced by *Exophiala* sp. isolates, which belong to the same OTU, but have different growth patterns in culture. Chapter 6 (Maciá-Vicente *et al*., 2016) comprises the description of *Exophiala radicis* as a new species, based on phylogenetic and micromophological description of cultures isolated in Chapter 1. Finally, in the General Discussion I discuss the findings of all studies aiming to answer the main questions of the thesis and provide recommendations for further research.

General Discussion

1. Diversity of root fungal endophytes in *Microthlaspi* spp.

This thesis sought to explore the fungal endophytic diversity found within the roots of *M. perfoliatum* and *M. erraticum*, across different locations and environmental conditions, with the aim to assess which fungal taxa can surpass the root surface and infect the roots endophytically.

1.1 Taxonomic diversity

In Chapter 1, the use of cultivation-based methods uncovered only cultivable taxa, which, as found in Chapter 2 with HTS, provided a good representation of the core root endophytic mycobiome of *Microthlaspi* spp. Even though the HTS method generated a larger amount of OTUs than the cultivation-based method, interestingly, both methods agreed on the dominance of just a few taxa within Pleosporales, Hypocreales and Helotiales. This observation supports the results of Chapter 1 and proves that the cultivation of roots was successful in assessing the presence, but also the abundance, of fungal taxa in most cases. Cultivation-based methods have been reported to favor the growth of specific fungi over the growth of slower-growing ones which are often not detectable, either because they are overgrown by the faster growers or because they require a long time to grow (Porras-Alfaro and Bayman, 2011). In this study, the detection of high abundances for particular OTUs, such as those related to *Fusarium* spp., which had faster growth in culture in comparison to others, could represent a potential bias inherent to the method. However, their large representation in the HTS dataset of Chapter 2, suggests that they are an important fraction of the endophytic mycobiome and, therefore, may hold potential

ecological significance. The implementation of both methods was indeed very useful to extract reliable results; this strategy has been proposed as the optimal approach to study diversity of fungi (Porras-Alfaro and Bayman, 2011). This is because, as opposed to other microbes such as bacteria, there is a large fraction of fungal species that can be grown in culture (Porras-Alfaro and Bayman, 2011). Importantly, the use of HTS was expected to uncover a large uncultivable diversity, considering the estimated large unexplored fungal diversity, especially in association with plants (Taylor *et al.*, 2014; Peay *et al.*, 2016). Indeed, a large number of taxa which had not been previously cultivated were detected, however, there was no indication of a systematic representation of uncultivable fungi that would imply potential symbiotic importance.

The large diversity uncovered by the use of both methods agreed with the general results observed. For example, the skewed microbial species abundance distribution, with few very abundant but many rare OTUs, is typical in fungal communities (Maciá-Vicente *et al.*, 2008, 2012; Unterseher *et al.*, 2011), and suggests a dominance of a few abundant fungi with respect to many rare ones. In addition, many taxa described here (specifically within Pleosporales and Hypocreales) were also found in other studies in the related hosts of *Arabidopsis thaliana* (García *et al.*, 2012; Keim *et al.*, 2014)and *M. perfoliatum* (Keim *et al.*, 2014), but also in other plants (Maciá-Vicente *et al.*, 2008, 2012). The dominance of Ascomycota over other phyla has been previously reported (Porras-Alfaro *et al.*, 2008) and it is indicative of a high specificity of ascomycetous fungi to endophytically colonize plant roots. For Basidiomycota, while their small representation found in *Microthlaspi* roots with cultivation-based methods in Chapter 1 can be attributed to their inability to grow on the specific medium used, the low abundance also found with HTS in Chapter 2, is indicative of their overall low representation in the roots of these hosts. A possible explanation for this could be that Basidiomycota are found more frequently in forest environments, developing ectomycorrhizal symbioses with woody plants, whilst *Microthlaspi* spp. used for this study were collected from disturbed areas next to roads and grasslands. One approach to test whether the low abundance of Basidiomycota is related to the low representation in the local environment, or to an active filtering by the host, would be to characterize the fungal diversity within the soil and evaluate the available pool of Basidiomycota species in the soil around the host plants' roots. Even so, in studies of endophytes associated with plant hosts in the Brassicaceae family, the abundance of Basidiomycota was also found to be low which could suggest an overall underrepresentation of this phylum (García *et al.*, 2012; Keim *et*

al., 2014). It is important to note, however, that the patterns of diversity described in this study relied solely on the sequencing of ITS rDNA regions. Despite the large diversity of Ascomycota being unravelled, it is possible that not all fungi are active members of the endophytic assemblages, but, in turn, may be in dormancy (Lennon and Jones, 2011). By targeting both DNA and RNA sequencing, it is possible to distinguish between which of the present taxa are metabolically active and those which are not and, therefore, not contributing to the function of the communities (Ofek *et al.*, 2014).

1.2. Findings for abundant and rare fungal endophytes

Even though OTU assignment is considered as a putative taxa delimitation and potentially biased taxonomic classification of microbes, the lack of intra-OTU variability in the five most abundant OTUs found with the use of multilocus sequencing and AFLP profiling in Chapter 3, indicates their suitability to delimit many fungal groups. Such low genotypic diversity was not expected, taking into account the widespread distribution of these fungi across Europe, but it has also been found for soil (Queloz *et al.*, 2011) and arbuscular mycorrhizal fungi (Rosendahl *et al.*, 2009). The geographic distance between populations was expected to induce the genetic diversification of these fungi due to ecological isolation (Giraud *et al.*, 2008). The genetic diversification in isolated fungal populations would imply sexual reproduction among individuals, homogenization of the population and genetic diversification due to the lack of gene flow (Rosendahl *et al.*, 2009). Alternatively, for fungi that do not develop reproductive structures, which is possibly the case for the fungal endophytes of this study, niche differentiation and adaptation to environmental conditions could induce genotypic variability within taxa (Rosendahl *et al.*, 2009). It is conceivable that those five fungal endophytic groups studied for possible undetected genetic heterogeneity in Chapter 3 but exhibited genetic homogeneity, are in contact via dispersal over long distances. This contact allows for a constant gene flow among populations and hinders genetic diversification. On the other hand, they may have adapted to the endophytic lifestyle within the specific host and, for this reason, they may need to maintain the same genetic background. However, the opposite findings for *Cadophora* sp. OTU006 are indicative of a strong, genotypic variation and gives rise to the consideration that these fungi may constitute a unique group. Apparently, these fungi follow a different biogeographical pattern in comparison

with other fungal groups. It is also possible that within OTU006, different species are looped together. Nevertheless, considering the pathogenic potential of these fungi, further studies on their diversification processes are needed. Greater focus should be given to explore the genetic variability of genes, relating to the metabolic activity and function of fungi (Aguilar-Trigueros *et al.*, 2014), as these may be more exposed to divergence due to environmental conditions and trait differentiation. In addition, applying HTS techniques (Branco *et al.*, 2015; Wyss *et al.*, 2016). 2016) may reveal fine-scale genetic variation among individuals due to the higher sensitivity of these methods. Whole-genome sequencing, for example, would be appropriate to detect genetic divergence, considering that this method describes variation in the whole genome. Finally, since this study covers populations found in different environmental conditions, it would also be useful to study the epigenetic differences amongst individuals which are caused due to the different environmental conditions (Foust *et al.*, 2016).

This thesis focused on assessing the phylogenetic background of the core endophytic mycobiome in hapter 1 and 2 and the most abundant taxa in hapter , despite there being a large diversity of fungi present. These taxa were chosen because their abundance was considered to be ecologically important and they were further studied for their effects on plant performance in Chapter 4, in comparison to the low-abundance taxa, the presence of which may be random. However, the abundance of a species is not always an indicator of ecological significance and functional dominance (Berry and Widder, 2014; Agler *et al.*, 2016). In fact, it has been suggested that rare species are an important part of microbial communities, significantly contributing to their function. Rare species are usually considered transient colonizers, with little effect on the plant, but their low abundance may also imply strong adaptation to a host organ. In addition, they may develop important interactions with other endophytic species and determine the structure of microbial communities, via their involvement on important metabolic processes within assemblages (Copeland *et al.*, 2015; Jousset *et al.*, 2017). One way to assess whether rare taxa affect the presence of other fungi, is by applying network analyses to identify the co-occurrence patterns among fungi (Pan and May, 2009; Agler *et al.*, 2016). Thus, the fungal groups found to be connected with the occurrence of multiple fungi, are likely to be important members of the assemblages (Berry and Widder, 2014).

1.3. Characterizing diversity based on functional, morphological and metabolic data

The taxonomic characterization of the fungal endophytic diversity in this thesis was based on the genetic similarity of the fungi. However, a more comprehensive, functional classification of the diversity, based on their functional traits, is necessary in order to elucidate the ecological roles of endophytes. The association of the fungal endophytic diversity with the ability of the taxa to grow in culture was an initial approach made towards shifting from the taxonomic description of communities to assessing their functional diversity. In fact, in Chapter 2, the culturability of fungi was used as a proxy to classify, functionally, the diversity detected in the broad spectrum of saprotrophs for those fungi which utilize carbon sources from non-living organic material and the biotrophic fungi dependent on the symbiosis with the host or other microbes (Bonito *et al.*, 2016). Results revealed that many of the taxa detected in the HTS were isolated by the cultivation of roots and that there was no detection of frequent OTUs unique for the HTS. Thus, we can conclude that the fungi which endophytically infect the roots of *Microthlaspi* spp. plants have saprotrophic activity. In addition, the absence of fungal groups phylogenetically related to biotrophic fungi, indicated that the uncultivated, rare OTUs were taxa exhibiting a slow-growing habit on agar medium rather than being biotrophs. A more comprehensive description of the functional diversity, however, is needed and this would include the investigation of the functional genes in relation to the transport of nutrients for symbiotic fungi or the enzymatic activity for carbon degradation in facultative saprotrophs (Aguilar-Trigueros *et al.*, 2014). Such an approach was followed in Chapter 4, where the production of several enzymes relating to root colonization were assessed.

Diversity analyses in this thesis were based on molecular tools. Nevertheless, an attempt to integrate morphological descriptions of fungal colonies was also carried out in Chapter 3. However, for the groups of fungi investigated, the morphological differences were not reflected in the genetic background. It is possible that these differences were caused by the presence of

mycoviruses, since other research on the effect of viruses on fungi has reported changes in the structure, color and growth of the mycelium, similar to the ones reported here (Ghabrial and Suzuki, 2009; Aoki *et al.*, 2009; Rohwer and Youle, 2011). This hypothesis should be investigated further since mycoviruses do not only induce morphological abnormalities in fungal cultures, but also influence their function and interactions with their host (Pearson *et al.*, 2009; Yu *et al.*, 2010). In addition, focus should be given to the micromorphological description of the colonies based on the shape and size of their spores, rather than on their macromorphological characteristics. In fact, such an approach was followed in Chapter 6. The detection of differential colony growth in growth medium for individuals of the same OTU, led to the multilocus phylogenetic analyses which resulted in the description of a new species, *Exophiala radicis*. In addition, the fact that the description of *E. radicis* was based more on the differences in the spore structure, rather than on the genetic variation, highlights the necessity of assessing micromorphological characteristics.

The variability in the production of metabolic compounds can also be used to delimit species and shift from taxonomical characterization to the description of metabolic diversity (Knapp and Kovács, 2016). This approach was followed in Chapter 5, where characterization of the metabolic profiles of endophytic fungi showed discrepancies in the structure of the exophillic acid and was found to be associated with differences in the colony morphology of isolates of *Exophiala* sp. The structural variation of the exophillic acid could represent a potential chemotaxonomic marker, which could serve exactly as a genetic marker in the taxonomic delimitation of individuals within the *Exophiala* genus. The establishment of chemotaxonomic markers could be, ultimately, a useful concept in the characterization of fungi, as structural variation of their chemical compounds could be attributable to differences in their metabolic profiles and their function within endophytic assemblages (Frisvad *et al.*, 2008).

2. Ecological determinants of root fungal endophytic assemblages

2.1 Factors affecting the structure of assemblages

The study of the ecological factors determining the composition of fungal endophytic communities included several types of variables, including geographic (location, altitude), climatic (temperature, precipitation, bioclimatic descriptors), physicochemical (soil chemistry and structure) and genetic (host plant genotype). These factors have been shown to be significant determinants of microbial communities. For example, several studies on foliar endophytic and soil fungal communities suggest that their structure is affected by temperature and precipitation (Arnold and Lutzoni, 2007; Hawkes *et al.*, 2011; Timling *et al.*, 2014; Tedersoo *et al.*, 2014; Cox *et al.*, 2016). Moreover, several studies revealed a significant effect of the host genotype on plant-associated communities (Bálint *et al.*, 2015, 2016; Sapkota *et al.*, 2015). Regarding soil variables, for several fungi it has been demonstrated that their presence is linked to soil nutrient availability. For example, helotialean fungi are abundant in soils with high nitrogen whilst mycorrhizal fungi form symbioses with plants under soil phosphorus deficiency (Read 1991; Read *et al.*, 2004). The structure of the root fungal endophytic assemblages was investigated in both the cultivation-based and the HTS studies (Chapters 1 and 2, respectively); these agreed in the patterns that they presented, where several spatial, bioclimatic and fewer soil factors were found to drive changes in the assemblages. Nevertheless, with deeper sequencing, described in Chapter 2, fine-scale variations were detected which revealed a stronger effect of soil conditions than the experiments in Chapter 1. However, as root fungal endophytic assemblages are in direct contact with the soil, a clearer effect of soil conditions was expected. Possibly the effect of soil can be seen only when the variation in soil is great enough to exceed the species' ranges of tolerance, as, for example, in saline environments (Maciá-Vicente *et al.*, 2012). In addition, the presence of the endophytes detected does not seem to be associated with the high or low availability of specific soil nutrients, as seen in mycorrhiza. This could be an indicator of the lack of symbiotic interactions between endophytes and plants in relation to the transfer of nutrients. Nevertheless, this lack of soil effect may be attributable to the ecology of the host plant. Indicatively, *Microthlaspi* spp. are pioneers

in growing in disturbed environments where nutrient availability is relatively high (Koch and Bernhardt, 2004) and are among the few plant species which do not develop symbioses with mycorrhizas.

When elucidating the processes underlying the structure of microbial assemblages, stochastic processes such as dispersal limitation and priority effects often influence the observed patterns (Maherali and Klironomos, 2007). For the endophytic assemblages studied here, the results do not support a strong dispersal limitation. However, it is possible that the assemblages are subject to strong priority effects, favoring the establishment of earlier colonizers as has been shown in other fungi (Hiscox *et al.*, 2015; Bahram *et al.*, 2016). This effect is particularly strong when fungi undergo competitive exclusion for the colonization of a resource and are not subject to ecological filtering, as, apparently, is the case in this system (Peay *et al.*, 2016). Priority effects, therefore, could also introduce the variation among endophytic assemblages, as the first colonizers may significantly affect the colonization success of the latter ones (Hiscox *et al.*, 2015).

The widespread distribution of the abundant OTUs and the detection of endemicity in the distribution of rare species, suggest that the differences in the assemblages' structure may be related to the effect of ecological factors on the distribution of rare fungi. Rare fungi may be subject to dispersal limitations due to their anatomical characteristics, however, this would imply the detection of highly similar assemblages among locations with geographic proximity, decreasing as the distance increases. This association, however, was supported only for short distances by the Mantel tests of Chapters 1 and 2, when testing how assemblages change with distance. On the contrary, it is more plausible that spatial factors, as well as the environment, may affect the distribution of the low-abundance fungi and determine their growth in a specific location, consequently determining their establishment within endophytic assemblages. Nevertheless, even if significant differences in the composition of assemblages were detected, rare fungi may have the same roles and, therefore, the overall function of the assemblages may remain similar.

It is important to emphasize that the host identity was not found to affect the structure of the assemblages, either in the cultivation-based study nor when using HTS. However, future studies on the diversity of different plant species in the same sampling site are necessary to

unravel the effects of host genotype in shaping the endophytic mycobiome. In fact, the host identity has already been shown to affect the composition of assmeblages of leaf-associated fungi (Bálint *et al.*, 2013; Wehner *et al.*, 2014) and bacteria in the rhizosphere (Ofek *et al.*, 2014). Such a study should focus on exploring the diversity on adjacent hosts which are both phylogenetically related but also very divergent. This could also help to assess whether the rare endophytic taxa are random colonizers or specialized to specific hosts in which they can be found as frequent colonizers (Joshee *et al.*, 2009; Yuan *et al.*, 2011). In addition, assessment of the seasonal variations in the structure of endophytic communities is also necessary, as it has been shown that these factors can significantly affect the succession of the microbiome in plants (Mandyam and Jumpponen, 2008; Scholtysik *et al.*, 2013; Copeland *et al.*, 2015). Since *Microthlaspi* spp. are annual plants, it would be interesting to assess whether the assemblages characterized are generalistic due to the short plant life cycle. On the other hand, if assemblages are subject to active differentiation through the life of the host, this could suggest that direct interactions between fungal endophytes and the host do take place.

The phylogenetic congruence of OTUs within the core endophytic mycobiome described in Chapter 2 was expected to reflect similar response to ecological variables. Interestingly, the lack of phylogenetic conservatism in response to spatial, bioclimatic and soil variables, shows that the genetic background of a fungus is not always an indicator of its preferences. On the contrary, in all three fungal orders analyzed, the overall effect of ecological conditions was highly variable, even for genetically closely related OTUs. The lack of phylogenetic conservatism among ecological gradients provides some preliminary evidence that endophytes are subject to competitive exclusion during the organization of their assemblages (Webb *et al.*, 2002). This is possibly a consequence of the functional traits being likely conserved among phylogenetically related species, increasing the competition between taxa for the same resource. Such forces may consequently lead to niche partitioning in order to decrease the competition, a concept which has been previously described for soil fungi within the phylum Ascomycota (Taylor *et al.*, 2014) and mycorrhizal fungi (Jarvis *et al.*, 2015). Nevertheless, it is important to consider that this analysis is based on the phylogenetic relationships of OTUs based on the ITS sequence similarity and, therefore, is not conclusive. While ITS is a good indicator for identifying preliminary genetic differences, phylogenetic analyses involving the characterization of more loci, as employed in Chapter 3, would be necessary. Possibly, the implementation of a

comprehensive phylogenetic analysis could reveal details of phylogenetic conservatism that have previously remained undetected.

This thesis provides a useful basis for future studies in order to elucidate the ecological processes governing the organization of endophytic assemblages and their impact on the host and the environment. The characterization of the core fungal OTUs within the endophytic communities of *Microthlaspi* spp. and their preservation in culture is a useful source that can be utilized for the selection of candidates in order to generate an artificial assembly with the most-represented fungi. Manipulation of the assembly can be made with the inclusion or exclusion of candidate fungal groups (Bever *et al.*, 2010; Bodenhausen *et al.*, 2014; Panke-Buisse *et al.*, 2015). These experiments, in concordance with theoretical studies based on simulations of communities, can facilitate the research on systems of a complex nature such as the endophytic assemblages. For the next level of research, investigation of fungal-bacterial interactions which are equally important in the endophytic assemblages (Berg *et al.*, 2015) is necessary, considering the current evidence on their active interactions with fungi (Frey-Klett *et al.*, 2011; Agler *et al.*, 2016). Finally, the integration of research on the chemical compounds and exudates shaping the chemical profile of the endophytic mycobiome is necessary to unravel the signalling processes taking place during the establishment of plant-endophyte interactions (Knapp and Kovács, 2016).

2.2. Distribution of fungal endophytes

The dominance and ubiquity of only six OTUs, reported in Chapter 1, was believed to be worthy of further investigation since these fungi appear to be highly adapted to the endophytic lifestyle in *Microthlaspi* spp and possibly have important symbiotic functions. The genetic homogeneity reported in Chapter 3, even after using several sensitive molecular methods to address intra-group genetic variability, is indicative of the ability of these fungi to disperse over long distances. The findings clearly support the Baas-Becking hypothesis, of the unconstrained ability of fungi to disperse, which contrasts with recent views on this subject (Taylor *et al.*, 2006; Peay *et al.*, 2010a; van der Gast, 2015). However, according to the results in Chapter 3, these fungi are also able to colonize and establish themselves in

different environments as has been shown previously for arbuscular mycorrhizal fungi (Hazard *et al.*, 2013) and other root-associated fungi (Queloz *et al.*, 2011). While the environmental conditions across the sampling sites were not drastically different, an effect on fungal distribution was expected since differences have been reported in other studies (Taylor *et al.*, 2006, 2008; Salgado-Salazar *et al.*, 2013; Cox *et al.*, 2016; Peay *et al.*, 2016). Possibly, the observed patterns of distribution are associated with the high specificity of these fungi to endophytically infect the hosts. If this is the case, then their distribution is more related to the presence of the host rather than to other environmental conditions. This hypothesis implies that through symbiotic interactions with the host, these fungi are able to withstand environmental stress and follow the distribution of their hosts. To test this hypothesis, it is necessary to assess the niche breadth of these fungi and their ability to colonize environments with drastically different environmental conditions, in the presence of the same and different plant hosts. Nevertheless, further assessment of their genetic background is needed by implementing the sequencing of molecular markers with higher genetic resolution than ITS.

Another possible reason for the ubiquity of the fungi studied in Chapter 3 may be the small size of their spores as well as their dispersal mechanisms which are able to utilize wind and water forces (Nelson *et al.*, 1994; Mims and Mims, 2004; Palmero *et al.*, 2010; Skjøth *et al.*, 2016). However, studies on the spore dispersal of Basidiomycota show that their spores do not extend unlimitedly (Peay *et al.*, 2010b). In addition, most of the endophytic fungi isolated in this study did not produce spores in culture, which may imply that the presence of these fungi in roots is solely in the form of hyphae. Thus, the mechanisms for the efficient dispersal of fungi may be related to the utilization of carrying agents, rather than the dispersal of fungi by spores. It is also possible that these fungi disperse via host dispersal through seeds. In this case, they may follow the distribution of their host, as for example *M. perfoliatum*, which experienced a postglacial migation from southern to northern Europe (Koch and Bernhardt, 2004). Nevertheless, this should also offer limited dispersal, as it has been shown that seeds in *Pinus* spp. are rather devoid of fungi; only a few fungi are capable of colonizing the interior of these seeds (Ganley and Newcombe, 2006). Animals may also facilitate as agents for fungal dispersal, acting as carriers of fungi. In addition, human movement and the expansion of agriculture as it has developed during the last centuries, may have contributed considerably to the movement of fungi (Rosendahl *et al.*, 2009). In any case, the processes determining the distribution of fungal endophytes needs to be better described, since the presence of

these fungi can have direct effects not only on the local fungal community, but also on the host plant. Importantly, with the dispersal of several fungal species, invasive genotypes can cause the expansion of fungal diseases with detrimental effects on crops (Brown and Hovmøller, 2002).

3. Function of root fungal endophytes

The diversity and ecological analyses of this thesis are a useful step to move from describing patterns in the composition of the root endophytic mycobiome to investigating the underlying processes (Weber and Agrawal, 2012) and to postulate hypotheses on the functions of fungal endophytes. The dominance of cultivable fungal taxa suggests that these fungal assemblages are mostly formed by fungi with a saprophytic lifestyle. However, the maintenance of a healthy state of the plant at the time of their collection indicates that these fungi likely act as facultative colonizers of the root system. Under specific conditions, as for example during plant senescence, these endophytes may switch to saprotrophy for exploitation of the decaying plant substrates as nutrients (Promputtha *et al.*, 2007; Porras-Alfaro and Bayman, 2011). In addition, the phylogenetic relatedness of the dominant endophytic fungi with known plant pathogens, reinforces the hypothesis that their interaction with the host may be potentially detrimental. The systematic absence of non-cultivatable fungi indicates that the proportion of biotrophic fungi might be low within the assemblages studied. This suggests that the endophytic mycobiome of *Microthlaspi* spp. is not composed by mutualists which were expected to be found in place of the absent biotrophic mycorrhizal fungi. Nevertheless, it is conceivable that they develop context-dependent interactions and that their function is related to the specific environmental conditions that they are subjected to (Kia *et al.*, 2017). In any case, as it has been stated before, the functional diversity of the endophytic mycobiome may be different from the taxonomic one (Peay *et al.*, 2016) and fungi belonging to traditionally saprotrophic groups may lack genes related to decomposition. An example is the ericoid mycorrhizae, which constitute a group of fungi within Helotiales, able to grow in culture, they have saprotrophic potential but they also positive effect on plants via the transfer of nitrogen and phosphorus (Read, 1996).

While solely taxonomic data are not conclusive on the functional traits of taxa, the co-occurrence of taxonomically distinct lineages, such as the OTUs of Plesporales, Hypocreales and Helotiales, in all endophytic assemblages studied, suggests that assemblages are likely to be structured by fungi with different functional traits. It is, therefore, conceivable that the assemblages maintain functional complementarity, in which, under strong competition between fungi of similar traits, divergent fungi are organized together and complement each other on the function of the whole assembly and contribute to increased ecosystem functioning (Maherali and Klironomos, 2007). Nevertheless, it would be interesting to investigate the degree of similarities between the functional traits among endophytic assemblages. It is possible that, despite the similar patterns of taxonomic diversity, the varying environmental conditions have caused divergence in the functioning of endophytes leading to functional plasticity (Peay *et al.*, 2016). Nevertheless, the lack of intra-OTU genetic variability among sampling sites suggests that fungal traits and, consequently, functions are conserved among assemblages (Peay *et al.*, 2016).

The presence of the same fungal endophytic groups across sampling sites implies that these fungi maintain specific functions which allow them to thrive in the roots of their hosts. It would also be interesting, therefore, to assess whether their systematic presence is as a result of their active selection by the host. In addition, we could hypothesize that a preferential growth of an endophyte in a specific environment may be linked to specific functions which are favored by the local ecological conditions. Such a pattern was found, for example, for *Alternaria* sp. OTU002 which was growing preferentially in southern rather than in northern locations as found in Chapter 1. This hypothesis, however, relies only on observations and further experiments are needed to prove its validity. Indeed, several beneficial effects have been reported for endophytic species such as the protection of their host from drought or soil nutrient deficiency (Rodriguez *et al.*, 2009). However, *in vitro* experiments using individual strains in Chapter 4, did not reveal any beneficial effects on plant performance. Thus, the outcome of plant-endophyte interactions is probably based on the endophytic mycobiome functioning synergistically, rather than on the behavior of individual endophytic species (Berg *et al.*, 2015). In fact, the current status of research on the plant-microbiome symbiosis stresses the importance in regarding the plant microbiome and the host as a holobiont rather than individual organisms (Vandenkoornhuyse *et al.*, 2015). In fact, this would mean that the recruitment of a large diversity of fungal endophytes by *Microthlaspi* hosts benefits both sides via the enrichment of the pool of functional genes

assisting the fitness of the holobiont (Vandenkoornhuyse *et al.*, 2015; Rosenberg and Zilber-Rosenberg, 2016). The consideration of plants as organisms tightly interconnected with their microbiome is becoming widely acknowledged in agriculture and plant breeding systems as this may help tackle the problem of crop loss due to severe attacks by fungal pathogens (Hacquard, 2016). The negative effects on plant performance, but also the increased pathogenecity of fungi, is considered to be the result of the disturbance of the balance between the different members of the plant microbiome and the host (Frey-Klett *et al.*, 2011; Hacquard and Schadt, 2015; Hacquard, 2016). Thus, the purpose of using fungi for the improvement of plant growth may require the combination of applying functionally useful fungal species together with maintaining a healthy holobiont, rather than just focusing on eliminating microbial infections themselves (Berendsen *et al.*, 2012; Bakker *et al.*, 2012).

4. Concluding remarks

The findings of this thesis are useful both in elucidating the ecological determinants of fungal endophytic assemblages in plant roots and in attempting to answer major questions on what factors affect the distribution of fungi (Jumponnen *et al.*, 2017). This study concludes that there is a large diversity colonizing endophytically the roots of *Microthlaspi* spp. plants, from few dominant and widespread saprophytic groups and many others that are rare and locally-restricted. The consistent detection of fungal groups within the orders Pleosporales, Hypocreales and Helotiales, hints at a specificity of these fungi to infect the roots of these hosts. However, taxa within these orders have divergent niches; this may be a consequence of niche partitioning in order to reduce the intra-group competition. Fungal endophytic assemblages are structured under the effect of the local environment; bioclimatic conditions having a stronger effect which seems to influence the presence of rare taxa rather than the abundant ones. Finally, the classification of fungal endophytes based on their ability to grow in culture, as well as their demonstrated parasitic effect, hints at a strong relationship of these fungi with saprotrophic activity. These fungi appear to adapt an endophytic lifestyle, in order to avoid competition within the highly diverse soil communities, or switching to utilize carbon by becoming saprotrophs after host root senescence

(Schlegel *et al.*, 2016). By describing these patterns, this thesis provides new insight on the processes underlying the structure of the endophytic mycobiome. Ultimately, it raises hypotheses on the functions of fungal endophytes which are useful for the experimental investigation of their roles in ecosystems.

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Anlage 1

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovierende KG: 20%
Co-Author MT: 30%
Co-Author JGMV: 50%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende KG: 70% cultivation of roots, fungal isolation and preservation, DNA extraction, amplification (PCR) and sequencing
Co-Author JGMV: 25% cultivation of roots, fungal isolation and preservation, DNA extraction, amplification (PCR) and sequencing
Co-Author SHK: 5% fungal isolation and preservation

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende KG: 50% collection of root and soil material, curation of fungal collection, collection of sequencing data, collection of bioclimatic data, design of figures
Co-Author JGMV: 30% collection of root and soil material, design of figures, submission of sequences to Genbank
Co-Authors TA, AKB, SP, XX, AÇ, MT: together 20% collection of root material

(4) zur Analyse und Interpretation der Daten

Promovierende KG: 50% diversity analysis, statistical analysis
Co-Author JGMV: 45% diversity analysis, statitical analysis
Co-Authors TA, SHK, MT: together 5%

(5) zum Verfassen des Manuskripts

Promovierende KG: 50%
Co-Author JGMV: 40%
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Zustimmende Bestätigungen der oben genannten Angaben:

_____	_____
Datum/Ort	Unterschrift Promovend
_____	_____
Datum/Ort	Unterschrift Betreuer
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The local environment determines the assembly of root endophytic fungi at a continental scale

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Summary

Root endophytic fungi are found in a great variety of plants and ecosystems, but the ecological drivers of their biogeographic distribution are poorly understood. Here, we investigate the occurrence of root endophytes in the non-mycorrhizal plant genus *Microthlaspi*, and the effect of environmental factors and geographic distance in structuring their communities at a continental scale. We sampled 52 plant populations across the northern Mediterranean and central Europe and used a cultivation approach to study their endophytic communities. Cultivation of roots yielded 2601 isolates, which were grouped into 296 operational taxonomic units (OTUs) by internal transcribed spacer sequencing of 1998 representative colonies. Climatic and spatial factors were the best descriptors of the structure of endophytic communities, outweighing soil characteristics, host genotype and geographical distance. OTU richness was negatively affected by precipitation, and the composition of communities followed latitudinal gradients of precipitation and temperature. Only six widespread OTUs belonging to the orders Pleosporales, Hypocreales and Helotiales represented about 50% of all isolates. Assessments of their individual distribution revealed particular ecological preferences or a cosmopolitan occurrence. Our findings support a strong influence of the local environment in determining root endophytic communities, and show a different niche occupancy by individual endophytes.

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Introduction

Plant roots establish symbioses with a large diversity of microorganisms, some of which are able to penetrate the outer root boundaries and constitute endophytic assemblages different from those in the surrounding rhizosphere and rhizoplane (Lundberg *et al.*, 2012). Although some are transient colonizers that enter the roots due to stochastic events, others present adaptations that allow them to persist for long periods confined in particular compartments, or to more effectively invade the tissues and establish an active metabolic interaction with the host (Hardoim *et al.*, 2008). A single plant might contain a complex assembly of root endophytic fungi (Vandenkoornhuyse *et al.*, 2002), and plants in all terrestrial ecosystems have these associations. They can reach considerable microbial loads (Maciá-Vicente *et al.*, 2012), thereby constituting an important cost to the host as photosynthetic carbon is diverted to the symbionts. In exchange, some endophytic mycorrhizae provide their host plants with benefits, most prominently assisting in the uptake of nutrients and water, or protecting against stress (Kiers and van der Heijden, 2006; Van Der Heijden *et al.*, 2008; Kiers *et al.*, 2011). Other endophytes constitute a unidirectional sink for plant resources and develop parasitic or pathogenic relationships of varying magnitudes (Tellenbach *et al.*, 2011; Keim *et al.*, 2014; Mandyam and Jumpponen, 2014). Through these processes, endophytic fungi contribute to the functioning of land ecosystems by modulating plant productivity and diversity, alongside their implication in the cycling of soil carbon.

The largest fraction of the endophytic mycobiome remains poorly characterized. Although endophytes are hypothesized to impact plant fitness, experimental work has been unable to assign decisive functions to most of them (Mandyam and Jumpponen, 2005; 2014; Newsham, 2011). Because the function of organisms is necessarily

linked to their habitat, their potential ecological roles can be inferred from their natural occurrence, from the identification of the ecological factors affecting their communities, and from understanding how they affect them. For instance, dominant plant species characteristic of major biomes associate with different types of mycorrhizae, which develop distinct symbiotic functions in relation to the specific soil properties (Read, 1991; Read *et al*., 2004). There is substantial evidence that non-mycorrhizal root endophytes also have preferences towards ranges of hosts and environments (for an extensive review see Sieber and Grünig, 2013). Their local or regional occurrence can be linked to environmental variables like soil type and biotic factors like host phylogeny (Maciá-Vicente *et al*., 2008a; 2012). However, knowledge of the large-scale biogeographic patterns of non-mycorrhizal root endophytes is very limited, at best.

The biogeography of organisms is driven by environmental, geographic and historical factors, together with features intrinsic to them such as their lifestyle, their dispersal capabilities, or their biotic interactions (Prosser *et al*., 2007). As with other microorganisms, fungi were assumed to occur ubiquitously owing to large population sizes and a nearly unlimited ability to disperse (Fitter, 2005). This implies that their diversity is high locally, but comparably low at larger scales because the same species occur across landscapes, as summarized by the tenet 'everything is everywhere, but, the environment selects' (Baas-Becking, 1934). Evidence challenging this view has accumulated and depicts a more complex scenario for the distribution of different fungal guilds (Taylor *et al*., 2006; Amend *et al*., 2010; Tedersoo *et al*., 2014; Van der Gast, 2015). Non-mycorrhizal root endophytes have been suggested not to follow a biogeographic pattern (Queloz *et al*., 2011), as opposed to other aboveground and root-plant symbionts (Arnold and Lutzoni, 2007; Kivlin *et al*., 2011; Tedersoo *et al*., 2012; U'Ren *et al*., 2012). This could indicate that different processes govern the diversity of different fungal functional groups (Tedersoo *et al*., 2012). Alternatively, this could be a consequence of the lesser efforts devoted to study the broad-scale patterns of root endophytes.

Here, we investigate the biogeographic distribution of non-mycorrhizal root fungal endophytes at a continental scale, and evaluate the effects of geographic distance, local environment and the biogeography of their hosts in their community composition, and in the occurrence of dominant phylotypes. As for the host plant, we focus on closely related members of the annual genus *Microthlaspi* F.K. Meyer (Brassicaceae), which were until recently included in the species *Microthlaspi perfoliatum* (L.) F.K. Meyer (Ali *et al*., 2015). These comprise both diploid and polyploid cytotypes that are morphologically similar, but phylogenetically dissimilar. Current data have shown that they represent two distinct species that form predominantly selfing populations (Ali *et al*., 2015). Assessments of the distribution of endophytes have often focused on several unrelated host plants that were not represented in all sampling sites (Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; Maciá-Vicente *et al*., 2008a; Herrera *et al*., 2010; 2013; U'Ren *et al*., 2012). Because host phylogeny is one of the main factors determining the composition of plant-associated communities (U'Ren *et al*., 2012; Wehner *et al*., 2014), focusing on one host with a widespread occurrence may allow for more accurate biogeographical inferences. *Microthlaspi* has a broad distribution over nearly all of Europe (Meyer, 2003), allowing for samplings across a wide range of environmental gradients. As most Brassicaceae, *Microthlaspi* also lacks classical mycorrhizal associations and alternative adaptations for the efficient capture of soil nutrients because it dwells in habitats where these are not limiting (Fitter, 2005). This could leave additional niches open to other root colonizers with different effects on the host.

The aim of this study is to unravel the broad-scale biodiversity patterns of root endophytes and identify their key ecological drivers. We use *Microthlaspi* as a model host system and rely on a cultivation approach to characterize its endophytic mycobiome. The collection of an extensive inventory of fungal cultures will warrant further phylogenetic and ecological studies on these endophytes and on their interaction with plants.

Results

Diversity of root endophytes

A total of 424 plants were processed for isolation of root endophytic fungi, originating from 52 populations distributed along an area spanning four parallels and five meridians (Table 1). Out of the total plants sampled, 414 (97.4%) yielded endophytic fungal growth in at least 1 of the 10 root pieces plated. We recorded 2601 fungal colonies developing from 2359 out of the total 4240 root pieces, accounting for an overall colonization percentage of 55.6% (i.e., the proportion of root pieces yielding at least one isolate), and an averaged colonization per population of 56.7 ± 18% (mean ± standard deviation).

The fungal isolates were grouped into 296 OTUs by sequencing of the internal transcribed spacer (ITS) rDNA region of a subset of representative pure cultures (Fig. 1A). On average we obtained 16.5 ± 6.3 OTUs per population of *Microthlaspi* (Fig. 1B). The overall number of operational taxonomic units (OTUs) obtained was below the maximum expected richness of 344.3 OTUs as assessed by Bootstrap analysis, and the 564.3 OTUs as assessed by the Chao estimator. This translates into an average value of 4 ± 1.8 or 11.7 ± 13.7 OTUs that went undetected in each plant population respectively

Table 1. Description of *Microthlaspi* populations studied in this work, and results of fungal colonization and diversity.

Country	Site	Coordinates	Elevation (m.a.s.l.)	Host's ploidy	n^a	Isolates	Colonization (%)[b]	Observed richness		Estimated richness		Diversity indices	
								S^c	Av. S^d	Boot[e]	Chao[f]	H'^g	J^h
Bulgaria	BG-007	42.50 N / 22.82 E	614	Polyploid	6	60	86.7 ± 13.7	17	5.2 ± 1.9	20.6 (2.3)	62 (30.1)	2.3	0.8
	BG-010	42.70 N / 22.83 E	770	Diploid	9	78	81.1 ± 19	18	4.6 ± 1.3	21.8 (1.7)	27.3 (8.8)	2.4	0.8
	BG-011	42.67 N / 22.84 E	740	Mixed	5	43	76 ± 8.9	14	4.2 ± 1.8	17.2 (2.3)	15.7 (2.2)	2.3	0.9
	BG-012	42.66 N / 22.81 E	773	Polyploid	9	66	67.8 ± 13	25	3.8 ± 1.5	31.8 (3)	38.2 (10.2)	2.9	0.9
	BG-013	42.63 N / 22.73 E	837	Polyploid	9	65	64.4 ± 19.4	23	4.6 ± 2.2	28.5 (3)	62 (30.3)	2.7	0.8
	BG-014	42.59 N / 22.72 E	711	Diploid	4	37	85 ± 10	14	4.8 ± 1	17.6 (2.1)	29 (12.8)	2.0	0.8
	BG-015	42.57 N / 22.69 E	685	Diploid	9	84	77.8 ± 15.6	28	5.6 ± 1.5	34.5 (3)	35.3 (5.7)	2.9	0.9
	BG-023	42.91 N / 22.83 E	621	Polyploid	6	52	80 ± 22.8	14	4.3 ± 1.9	16.3 (2)	14.5 (1)	2.3	0.9
Germany	D-100	49.54 N / 09.34 E	415	Polyploid	10	82	69 ± 12.9	31	5.4 ± 2.2	38.8 (3.6)	48 (10.7)	2.8	0.8
	D-101	49.68 N / 10.00 E	278	Diploid	10	48	44 ± 29.5	20	3.4 ± 2.1	24.8 (2.8)	47.5 (22.7)	2.7	0.9
	D-102	49.45 N / 09.82 E	281	Diploid	10	32	29 ± 24.2	22	3.1 ± 3.2	28.3 (3.8)	46 (16.4)	2.9	1.0
	D-103	49.27 N / 09.84 E	299	Diploid	10	40	39 ± 26	21	3 ± 1.5	27.4 (2.7)	89 (48.6)	2.6	0.8
	D-104	48.61 N / 09.53 E	515	Diploid	10	51	41 ± 23.3	18	3.4 ± 1.6	22.5 (2.4)	40 (17.4)	2.3	0.8
	D-105	48.55 N / 10.12 E	481	Diploid	10	42	40 ± 13.3	23	3.6 ± 0.8	28.5 (2.3)	32.4 (7.2)	3.0	1.0
	D-11a	50.37 N / 07.22 E	504	Diploid	10	73	61 ± 24.7	20	4.1 ± 1.9	24.6 (2.8)	24 (3.9)	2.5	0.8
	D-11b	50.37 N / 07.22 E	504	Diploid	10	61	55 ± 21.2	17	3.7 ± 1.6	20.1 (1.7)	20.8 (4.2)	2.5	0.9
Spain	ES-001	38.04 N / 02.48 W	1630	Polyploid	10	54	50 ± 20	20	3.7 ± 1.2	25.4 (2.2)	46 (20)	2.5	0.8
	ES-002	38.05 N / 02.54 W	1612	Polyploid	10	58	50 ± 20.5	15	3.7 ± 1.6	17.7 (1.4)	25.5 (10.5)	2.3	0.8
	ES-003	38.09 N / 02.56 W	1253	n.d.	10	65	60 ± 18.3	21	3.5 ± 1.6	27.1 (2.6)	34.2 (10.2)	2.3	0.8
	ES-004	37.97 N / 02.45 W	1204	Polyploid	10	85	80 ± 15.6	17	3.6 ± 1.2	20.9 (2)	21.7 (4.5)	1.9	0.7
	ES-005	37.14 N / 03.48 W	1351	Polyploid	10	47	46 ± 23.7	11	2.2 ± 0.9	13.3 (1.6)	11.3 (0.7)	2.1	0.9
	ES-006	37.13 N / 03.43 W	1669	Polyploid	10	59	57 ± 14.9	11	2.9 ± 0.7	13 (1.3)	14.3 (4.1)	1.9	0.8
	ES-010	42.81 N / 04.25 W	1055	Polyploid	10	64	59 ± 18.5	24	3.3 ± 0.8	31.1 (2.5)	69.3 (31.8)	2.5	0.8
	ES-012	42.87 N / 04.15 W	1305	Polyploid	10	62	59 ± 27.7	17	3.2 ± 1.6	20.9 (2.3)	22.3 (5.4)	2.4	0.8
France	F-001	47.41 N / 06.56 E	285	Diploid	10	36	33 ± 25	13	2.2 ± 1.1	16.1 (1.8)	18 (5.5)	2.2	0.9
	F-002	47.14 N / 06.20 E	553	Diploid	10	45	43 ± 15.7	17	3 ± 0.7	20.8 (1.8)	18.3 (1.7)	2.6	0.9
	F-004	47.03 N / 06.33 E	699	Polyploid	10	27	27 ± 13.4	12	2.2 ± 1.2	15 (1.7)	26 (13.1)	2.0	0.8
	F-007	47.11 N / 06.07 E	543	Diploid	9	64	67.8 ± 18.6	16	3.8 ± 1.3	19.1 (1.6)	16.8 (1.3)	2.2	0.8
	F-008	47.08 N / 06.07 E	533	Diploid	9	32	34.4 ± 25.1	11	1.9 ± 1.1	14.2 (1.6)	12.5 (2.2)	2.0	0.8
	F-009	47.18 N / 05.46 E	216	Polyploid	9	77	75.6 ± 14.2	18	4.6 ± 1.6	22.3 (2.1)	23.6 (5.3)	2.3	0.8
	F-010	47.20 N / 05.43 E	198	Diploid	9	56	60 ± 17.3	18	3.8 ± 1.1	22.3 (2.1)	30 (10.7)	2.4	0.8
	F-011	47.32 N / 04.60 E	446	Polyploid	6	29	48.3 ± 23.2	8	2.2 ± 1	10 (1.5)	18 (10.1)	1.3	0.6
	F-013	47.30 N / 03.59 E	215	n.d.	6	35	55 ± 18.7	16	4 ± 1.5	19.7 (2.1)	38.5 (19.2)	2.5	0.9
	F-014	47.19 N / 01.20 E	121	Polyploid	7	62	75.7 ± 5.3	16	4.1 ± 1.1	19.6 (1.8)	18 (2.6)	2.3	0.8
	F-015	46.41 N / 00.22 E	112	Polyploid	9	42	43.3 ± 24.5	26	3.7 ± 2.2	33.5 (4.1)	48.7 (14.9)	3.1	0.9
	F-021	44.58 N / 05.38 E	1260	Polyploid	7	65	74.3 ± 21.5	17	5 ± 2.3	20.1 (2.1)	20.8 (4.2)	2.3	0.8
	F-023	44.49 N / 05.44 E	1095	Polyploid	9	59	57.8 ± 12	30	5 ± 1.7	37.5 (3.8)	47.1 (11.5)	3.2	0.9
	F-024	44.50 N / 05.42 E	734	Diploid	7	42	55.7 ± 23.7	20	4 ± 1.9	25 (2.9)	31.3 (9.5)	2.8	0.9
Greece	GR-001	39.81 N / 20.77 E	1065	Polyploid	10	87	82 ± 13.2	18	4.1 ± 1.9	22.2 (2.6)	33 (12.8)	2.1	0.7
	GR-002	38.94 N / 21.76 E	1410	Polyploid	10	71	61 ± 16	24	4.1 ± 1.4	29.8 (2.6)	37.2 (10.2)	2.7	0.9
	GR-003	38.91 N / 21.74 E	1283	Polyploid	10	57	55 ± 25.5	15	2.8 ± 1.5	18.3 (1.7)	17 (2.6)	2.1	0.8
	GR-004	38.91 N / 21.83 E	905	Polyploid	8	54	62.5 ± 17.5	20	3.9 ± 1.7	24.9 (2.7)	27.5 (6.3)	2.6	0.9
Croatia	HR-021	44.16 N / 15.58 E	795	Diploid	7	12	15.7 ± 17.2	8	1.4 ± 1.4	10.2 (1.4)	11.3 (4.1)	2.0	1.0
	HR-022	44.19 N / 15.52 E	574	Diploid	8	57	61.3 ± 24.2	17	4.3 ± 2	20.5 (2.1)	17.4 (0.9)	2.6	0.9
	HR-023	44.24 N / 15.54 E	760	Polyploid	4	12	27.5 ± 9.6	7	2.3 ± 1	8.9 (1.1)	12 (5.9)	1.7	0.9
	HR-025	44.46 N / 15.40 E	755	Diploid	10	47	46 ± 32	11	2.1 ± 1.2	14.1 (2.1)	18.5 (8.1)	1.6	0.6
	HR-028	44.59 N / 15.44 E	525	Diploid	8	14	17.5 ± 28.7	5	0.9 ± 1.1	6.4 (1.1)	8 (4.4)	1.3	0.8
Turkey	T-024	38.33 N / 30.64 E	1101	Polyploid	3	25	66.7 ± 15.3	9	5.3 ± 2.3	10.3 (1.6)	9.3 (0.9)	2.0	0.9
	T-025	38.39 N / 30.67 E	1180	Polyploid	3	17	53.3 ± 30.6	4	2 ± 1	4.9 (0.9)	7 (4.3)	0.7	0.5
	T-026	38.57 N / 30.58 E	1166	Polyploid	3	34	93.3 ± 11.5	5	3.7 ± 1.5	5.4 (0.7)	5 (0.4)	1.3	0.8
	T-027	38.79 N / 30.28 E	1105	Polyploid	3	20	50 ± 26.5	9	4.3 ± 2.3	10.6 (1.6)	11 (2.9)	2.0	0.9
	T-028	38.86 N / 30.00 E	1210	Polyploid	3	15	46.7 ± 41.6	8	3 ± 3	10.1 (2.3)	11.3 (4.1)	1.8	0.9

a. Number of plants sampled.
b. Percentage of root pieces yielding at least one fungal colony (±SD).
c. Overall observed OTU richness.
d. Mean observed OTU richness (±SD) across plant individuals.
e. Bootstrap incidence-based richness estimator (SE).
f. Unbiased Chao abundance-based richness estimator (SE).
g. Shannon's diversity index.
h. Pielou's evenness index.
n.d., not determined. SD, standard deviation; SE, standard error.

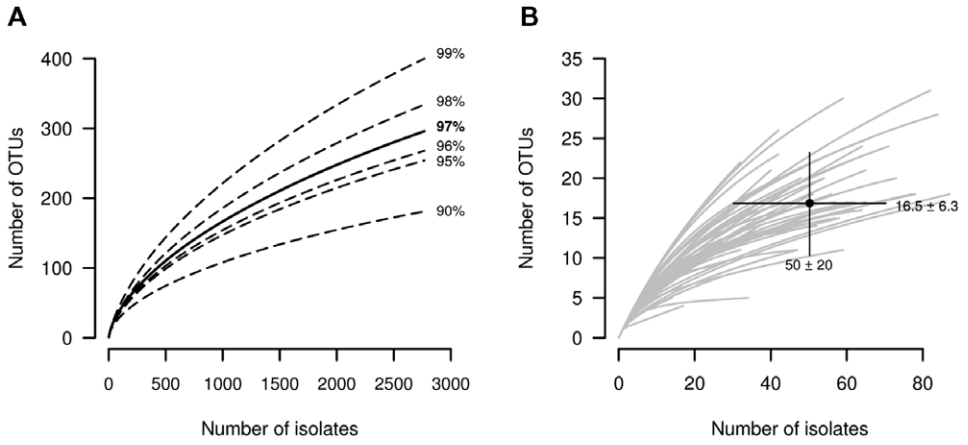

Fig. 1. Rarefaction curves of OTU accumulation with sampling effort, consisting of the total number of isolates developing from root pieces plated in a cultivation medium.
A. Accumulation curves for the entire study showing the effect of different sequence similarity thresholds for OTU definition.
B. Accumulation curves for individual *Microthlaspi* populations. The point with error bars indicate average and standard deviation for the number of isolates obtained per population (*x* axis), and number of OTUs per population (*y* axis), based on individual plant values.

(Table 1). The lack of saturation of the fungal diversity was confirmed by rarefaction curves calculated for the overall survey and for every individual plant population, which in most cases failed to reach an asymptote even when using similarity thresholds to define OTUs as low as 90% (Fig. 1).

Taxonomic classification of isolates

OTUs were classified at varying taxonomic precision by comparing ITS sequences with reference databases. They were assigned to 16 fungal orders, most of them within the Ascomycota (95%; Table S1). The Pleosporales and Hypocreales were the most represented orders, both in terms of the number of OTUs (43.2% and 19.6% respectively) and of the frequency of counts (Fig. 2A). The order Helotiales followed with 14.9% of the OTUs and a frequency of 11.6% of the colony counts, whereas the remaining orders were marginally represented (Fig. 2A; Table S1). Only six OTUs accounted for 50% of the isolates recorded (Fig. 2B). Three of these could be assigned to the order Hypocreales, two within the genus *Fusarium* – with affinities to the species *Fusarium tricintum* and *Fusarium avenaceum* (OTU001) and *Fusarium oxysporum* (OTU003) – and one within the genus *Ilyonectria* (OTU005). Another two of these OTUs belonged to the Pleosporales, the most abundant (OTU002) within the genus *Alternaria* – with close affinity to *Alternaria tellustris* – and another (OTU004) within *Pyrenochaeta* – with closest BLAST hits on *Pyrenochaeta lycopersici*. The sixth OTU in abundance (OTU006) was classified as *Cadophora* sp. Apart of their overall frequency, these OTUs had a widespread distribution and occurred in most plant populations, often representing an important proportion of communities (Fig. 2C). They were followed in abundance mostly by members of the Hypocreales and Pleosporales (Table S1). The remaining OTUs were in general infrequent, with 161 of the total 296 (54.4%) represented by a single isolate, and 47 (15.9%) and 21 (7.1%) by 2 and 3 isolates respectively.

Effect of environmental factors on endophytic diversity

Fungal assemblages differed significantly across populations in OTU richness ($H_{51} = 135.2$, $P < 0.001$), Shannon's diversity ($H_{51} = 121.2$, $P < 0.001$) and Pielou's evenness ($H_{51} = 93.1$, $P < 0.001$; Table 1). We compared endophytes' richness and diversity across environmental factors by using plant averages to correct for the different sampling sizes at each site (Table 1). None of these variables was significantly correlated with latitude (Fig. 3A), but linear regression showed a strong negative relationship of richness and diversity ($P < 0.002$) with various factors related to precipitation (Fig. 3B). These included annual precipitation at each site (Fig. 3B), precipitation of the wettest month, and precipitation of the wettest and coldest quarters of the year. Soil physico-chemical variables had no significant relationships with either richness or diversity of endophytic communities.

Effect of environmental factors on community structure

The unconstrained non-metric multidimensional scaling (NMDS) ordination of Horn–Morisita distances (stress: 20%) revealed a clear structure of endophytic communities along a latitudinal gradient (Fig. 4A). Differences among sites were significant when country of origin or climatic region were used as grouping variables in permu-

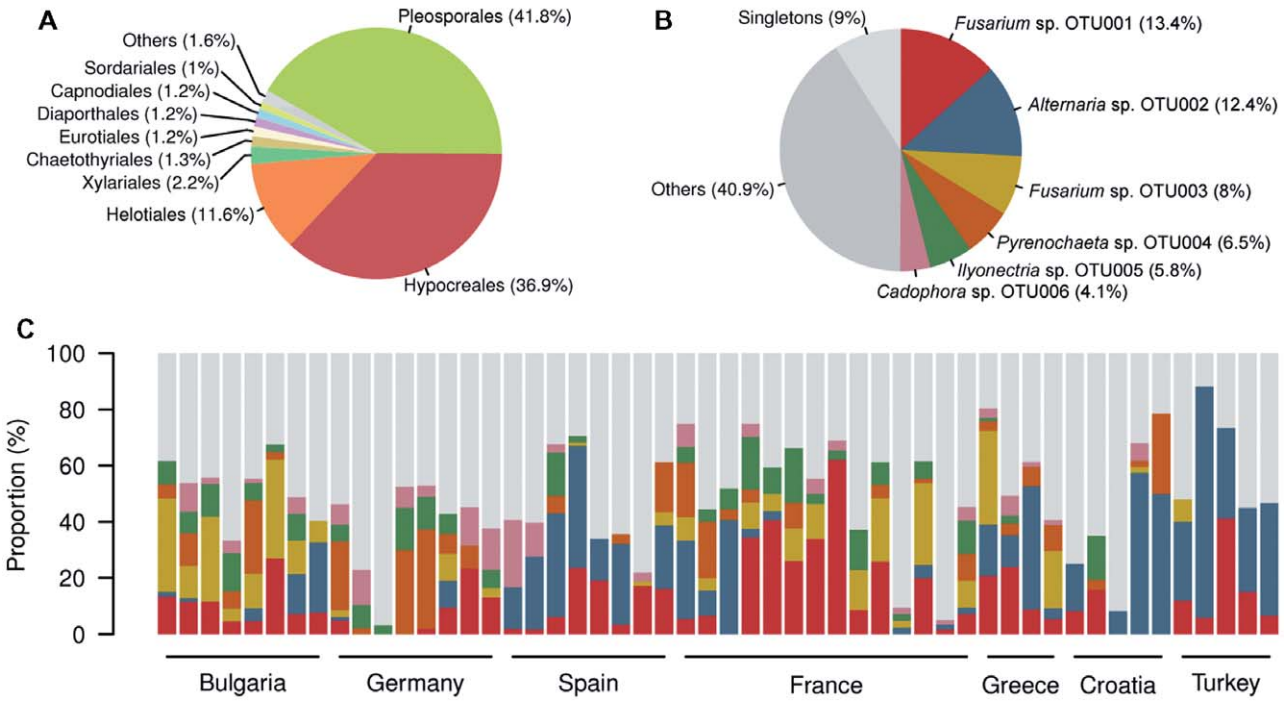

Fig. 2. Relative proportion of fungal taxa among the total number of endophytic isolates.
A. Proportion of isolates belonging to the most frequent fungal orders.
B. Proportion of isolates belonging to the most frequent OTUs.
C. Relative proportion of dominant OTUs across plant populations. Colours as in (B).

tation analysis of variance (PERMANOVA) ($P < 0.001$). All environmental variables retained in the forward selection as potential descriptors of communities had significant correlations with the ordination of sites and were strongly collinear with the latitudinal axis (Fig. 4A). Among these, the only soil variable with a certain degree of correlation with communities was Mg content (pseudo-$F_{40} = 1.8$, $P = 0.046$). We explored other variables not included in the forward selection in an attempt to explain variation in the axis perpendicular to latitude and found that the overall degree of endophytic colonization was the best fitting (pseudo-$F_{51} = 5.7$, $P = 0.001$; Fig. 4A).

An assessment of the distribution of the most common orders showed distinctive patterns of occurrence of Hypocreales in contrast to both Pleosporales and Helotiales (pseudo-$F_{57} = 1.8$, $P = 0.002$). Hypocreales tended to accumulate in communities leftwards in the ordination plot, perpendicular to the main axis of influence of environmental factors and positively correlated with overall colonization (Fig. 4B). Pleosporales and Helotiales on the other hand did not show a clear preference towards any factor.

Variation partitioning was used to assess the individual effect of climatic, spatial and host-related variables on the

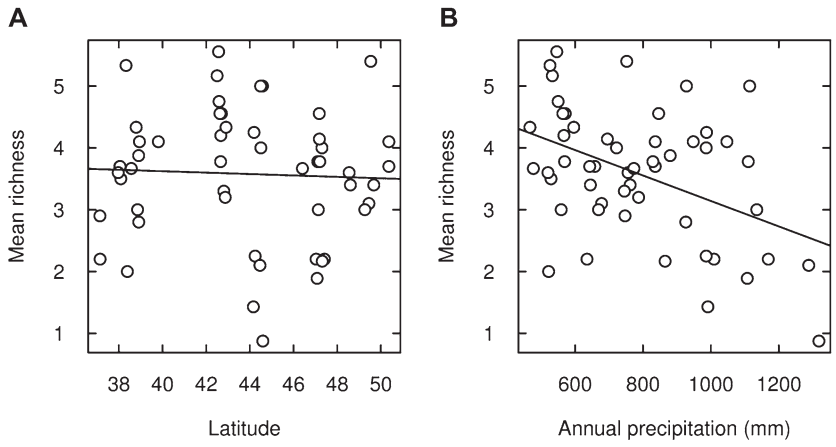

Fig. 3. Relationship between OTU richness in each *Microthlaspi* population, calculated as the average richness observed in each plant of the populations, and the respective latitude (A) and mean annual precipitation (B). Lines denote the linear regression model of interaction between both variables.

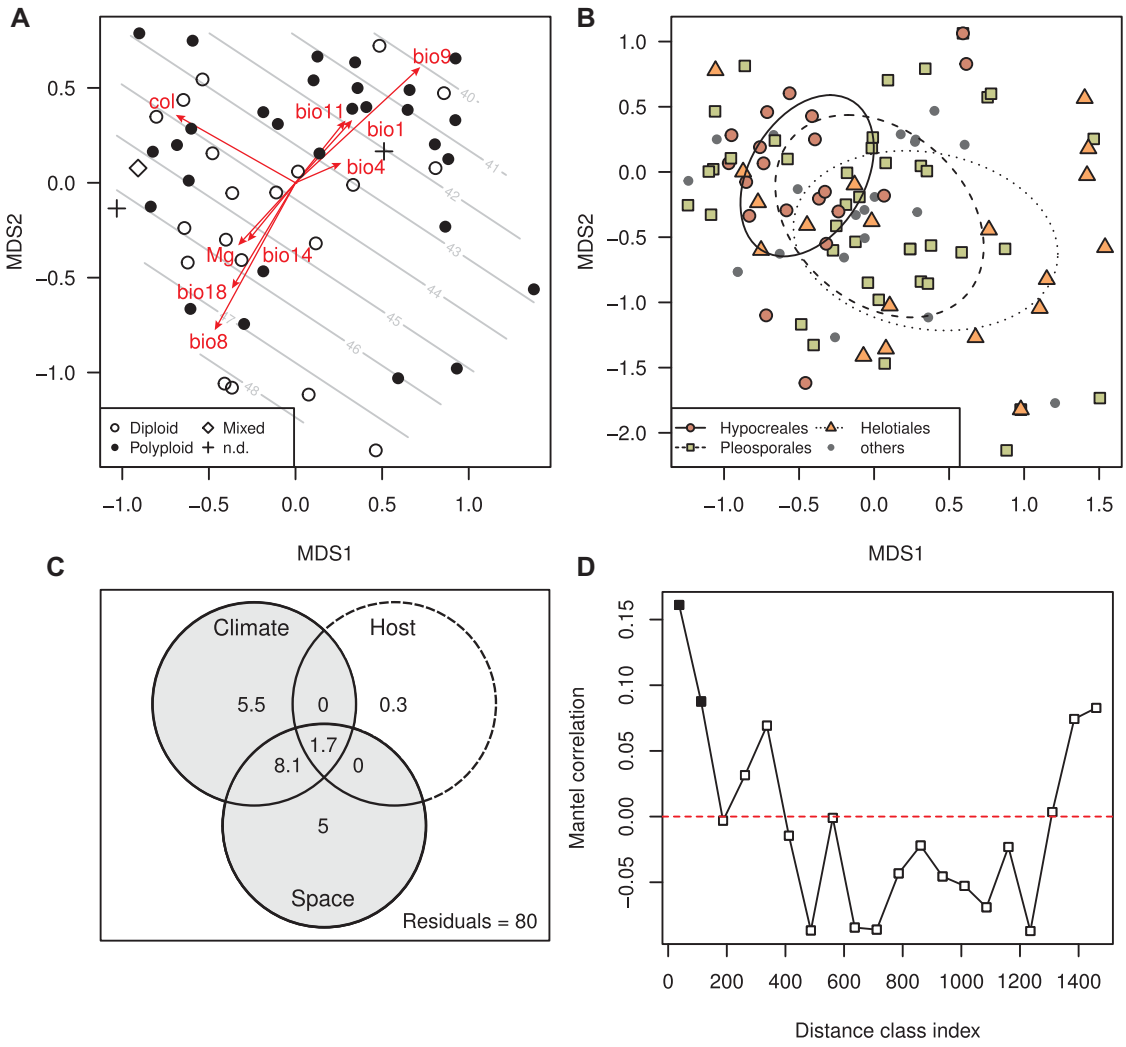

Fig. 4. Effect of ecological factors in whole-community structure of root endophytes.
A. Unconstrained non-metric multidimensional scaling (NMDS) analysis of communities displaying distances among populations, and depicting the relative influence of selected variables (arrows). The latitudinal gradient is represented as surface lines.
B. Species scores of the NMDS ordination in (A), highlighting the three dominant fungal orders. Ellipses delimit 95% confidence intervals around the mean values for each order.
C. Partition of the community variance into a climatic, a spatial and a host component. The numbers inside the sections indicate the percentage of the variation explained. Grey sectors with solid line indicate that the values comprised are significant ($P < 0.05$), whereas the value in an empty sector with dashed lines is not significant.
D. Mantel correlogram showing Mantel correlations among communities across distance classes. Solid symbols denote significant ($P < 0.05$) correlations for each class. Comparisons beyond 1500 km were not calculated due to the low number of samples included beyond this distance.
Abbreviations: bio1, annual mean temperature; bio4, temperature seasonality (standard deviation); bio8, mean temperature of wettest quarter; bio9, mean temperature of driest quarter; bio11, mean temperature of coldest quarter; bio14, precipitation of driest month; bio18, precipitation of warmest quarter; col, mean colonization percentage per population; Mg, magnesium soil content.

structure of endophytic communities (Fig. 4C). These three components explained 20% of the variance, and each accounted for a significant proportion individually according to pseudo-*F* tests ($P < 0.05$). However, the host predicted only a 1.7% of the overall variation ($P = 0.02$), indistinguishable from the contribution by climate or space. Each of the climatic and the spatial components explained individually around 15% of the total community variance, of which about 10% was jointly attributed to both categories (Fig. 4C). Sampling size accounted for a 3.3% of the overall variation, of which 2.4%, 1.8% and 1.1% were undistinguishable from the effect of climatic, spatial and host variables respectively. Sampling size alone explained a 0.7% ($P = 0.15$) of community variance.

Geographic distance had no overall effect on the similarities among communities ($R = 0.02$, $P = 0.32$). To investigate a potential effect across distance classes, we built a multivariate Mantel correlogram (Fig. 4D), which showed

a patchy distribution of communities separated by up to 115 km ($P < 0.002$).

Effect of ecological factors on individual endophyte populations

Maps of OTU occurrence and variance partition of individual fungal populations showed distinctive patterns in their distribution (Fig. 5). Occurrences of *Fusarium* sp. OTU001 (Fig. 5A) and *Pyrenochaeta* sp. OTU004 (Fig. 5D) were unaffected by the ecological components considered, and the models for their distribution were not significant. *Alternaria* sp. OTU002 (Fig. 5B) and *Ilyonectria* sp. OTU005 (Fig. 5E) showed clear but opposed latitudinal gradients of occurrence, mostly driven by confounding climatic and spatial factors. *Cadophora* sp. OTU006 was the only dominant endophyte the occurrence of which was consistent with a local distribution determined by soil factors, especially pH and Mg content (Fig. 5F). In this case, a fraction (5%) of the soil component overlapped with a significant effect ($P = 0.002$) of the sampling size. The contribution of the individual ecological variables to the occurrence of each OTU is shown in Table 2.

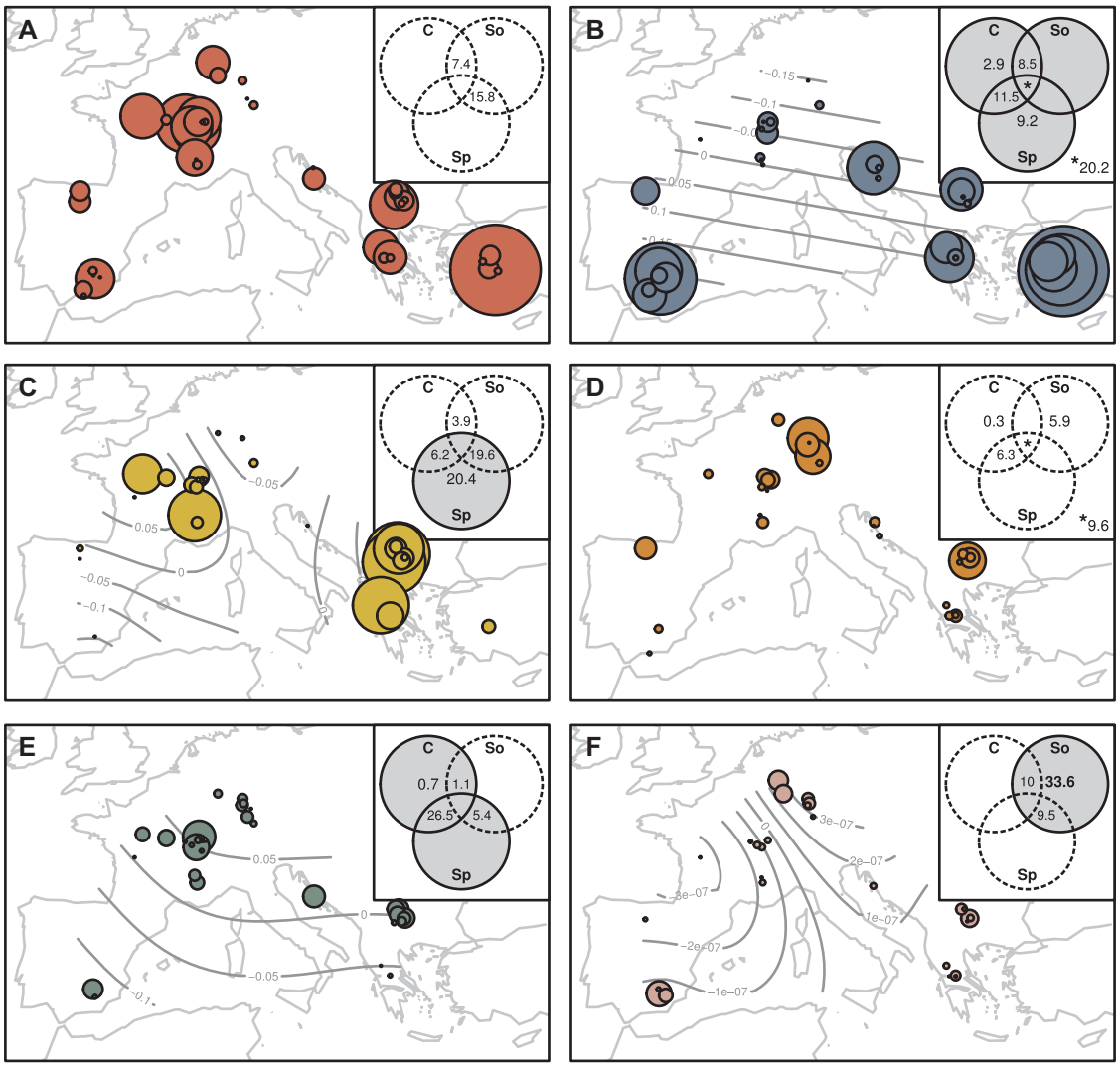

Fig. 5. Distribution and frequency of the six dominant OTUs across the sampling area: *Fusarium* sp. OTU001 (A), *Alternaria* sp. OTU002 (B), *Fusarium* sp. OTU003 (C), *Pyrenochaeta* sp. OTU004 (D), *Ilyonectria* sp. OTU005 (E) and *Cadophora* sp. OTU006 (F). Bubble sizes indicate relative frequency for each OTU at every location, and surface lines represent the fitted scores of redundancy analysis, depicting the variation explained by significant ecological components (samples from Croatia and Turkey not included in models, due to missing data on soil properties). Insets represent the variation partitioning results in a climatic (C), a spatial (Sp) and a soil (So) component. Grey sectors with solid line indicate significant values ($P < 0.05$), and empty sectors with dashed lines are not significant. Sectors without numbers indicate no variance explained at all.

Table 2. Variance partitioning for the occurrence of individual dominant endophytes into ecological categories of factors, including climatic, soil and spatial components.

		OTU001		OTU002		OTU003		OTU004		OTU005		OTU006	
Component	Factor[a]	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*
Climate	Annual temperature range (Bio 7)	–	–	0.2	0.002	–	–	–	–	–	–	–	–
	Mean temperature of wettest quarter (Bio 8)	–	–	0.19	0.004	–	–	–	–	–	–	–	–
	Mean temperature of driest quarter (Bio 9)	–	–	0.25	0.002	–	–	–	–	0.26	0.002	–	–
	Precipitation seasonality (Bio 15)	–	–	0.24	0.001	–	–	–	–	0.22	0.002	–	–
	Precipitation of warmest quarter (Bio 18)	–	–	0.13	0.015	–	–	–	–	0.23	0.002	–	–
Soil	Magnesium soil content (Mg)	–	–	0.14	0.01	–	–	–	–	–	–	0.24	0.003
	pH	–	–	–	–	–	–	–	–	–	–	0.26	0.001
	Latitude	–	–	0.36	0.001	–	–	–	–	0.22	0.003	–	–
Spatial[b]	MEM1	0.11	0.027	–	–	–	–	–	–	0.11	0.024	–	–
	MEM2	–	–	0.21	0.003	0.1	0.03	0.08	0.04	0.21	0.003	–	–
	MEM3	–	–	–	–	0.21	0.004	–	–	–	–	–	–
	MEM5	–	–	–	–	0.08	0.038	–	–	–	–	–	–

a. Ecological factors explaining a significant fraction of the occurrence of at least one fungal OTU, selected via a forward selection of all factors in this study.
b. MEMs (Moran's Eigenvector Maps) represent spatial structures in the data not explained by measured variables.

Co-occurrence and in-plate interactions of dominant endophytes

We found positive co-occurring patterns involving OTU001, OTU003 and OTU005, among each other and between OTU004 and OTU005 (Fig. 6). Relations among OTU001, OTU002 and OTU003 were strongly affected by a positive spatial autocorrelation, but the significance and magnitude of the relations persisted after correcting the spatial effect in linear regressions (slope = 0.30, $P = 0.014$ for the OTU001-OTU003 interaction). There was a strong negative correlation between OTU002 and OTU005 ($P < 0.001$), but this could not be linked with antagonistic interactions in culture (Fig. 6). Only *Pyrenochaeta* sp. OTU004 presented a consistent presence of inhibitory halos with other colonies. Both *Fusarium* strains tended to overgrow other fungi via direct contact between colonies because of their fast radial growth.

Discussion

The diversity and structure of fungal assemblages within roots of *Microthlaspi* were largely determined by the local environment to which plants were subjected. Of the ecological variables directly measured, climatic rather than soil conditions were the best descriptors of the broad-scale structure of endophytic communities. They were also strongly influenced by other factors that were lumped in the so-called spatial effect. This explains non-random spatial structures of the data not accounted for by the variables measured, and might include processes of environmental, historical or biological nature (Peres-Neto and Legendre, 2010; Dray *et al*., 2012). In contrast to the climatic and spatial effect, geographic distance among locations had a negligible influence in defining the composition of communities. The pattern arising when examining the turnover of OTUs across distance classes supported such view because communities that were close or very far away had greater commonalities than communities within intermediate distances. An effect purely due to distance and consequently to a limited dispersal, on the contrary, would imply a steady decay in community similarity with distance (the distance–decay relationship; Green *et al*., 2004; Peay *et al*., 2007).

In line with previous studies (Green *et al*., 2004; Amend *et al*., 2010; Queloz *et al*., 2011; U'Ren *et al*., 2012), our results do not reject the Baas–Becking hypothesis of a ubiquitous dispersal for fungi. Our findings are somewhat surprising because many root endophytes commonly lack specialized structures for dispersal in culture or field conditions (Jumpponen and Trappe, 1998; Sieber, 2002; Addy *et al*., 2005; Maciá-Vicente *et al*., 2008a), and even those able to produce spores have important constraints hindering their long-range dissemination (e.g., due to

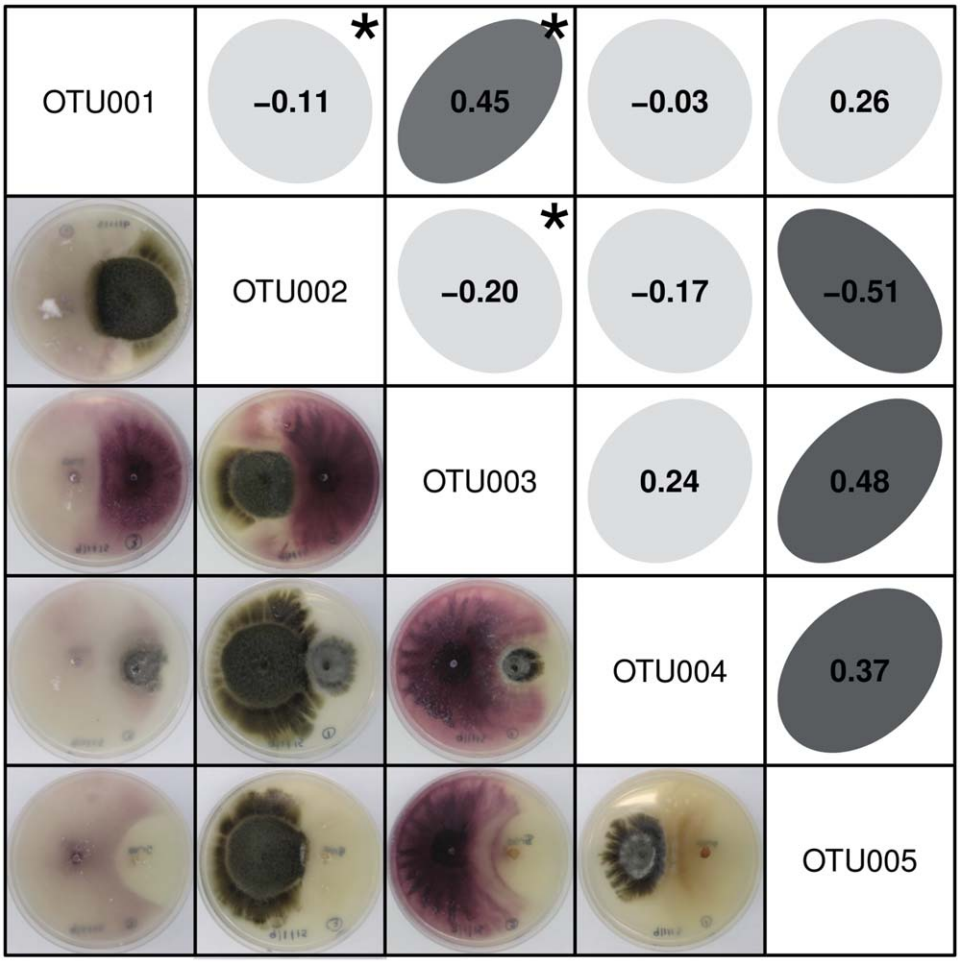

Fig. 6. Co-occurrence patterns and *in vitro* interactions among the five most frequent endophyte OTUs. Boxes with ellipses show the magnitude of the correlation between the co-occurrence of OTU pairs in roots of *Microthlaspi* populations. Values and ellipticity represent Spearman's ρ, and darker ellipses denote significance at $P < 0.05$. Asterisks indicate relationships with a significant autocorrelation, which were further assessed by spatial autoregressive models. Images in the lower diagonal represent interaction between colony pairs in dual culture assays. OTUs in each row are shown to the right in each interaction.

structural characteristics of the spores or their release points; Peay *et al*., 2010). Alternative mechanisms for their efficient dispersal must therefore exist. The hitchhiking with host dispersal could be relevant for endophytes that colonize the plant systemically and reach the seeds or fruits, but this mechanism cannot account for the majority of root endophytes that are restricted to below-ground plant organs (Rodriguez *et al*., 2009; Herrera *et al*., 2010; Maciá-Vicente *et al*., 2012). Other mechanisms of dissemination could imply animal transportation of plant material via herbivory and deposition, especially for some fungal groups that develop resistance structures within the plant tissues like microsclerotia (Currah *et al*., 1993; Porras-Alfaro *et al*., 2008), or processes in common with soil-borne fungi, like wind transportation by carrier soil particles, adhesion to invertebrates or spore washings (Dix and Webster, 1995).

Two considerations have to be taken into account with respect to the ubiquitous occurrence of root endophytes found in this study. First, our sampling could not achieve a complete description of the fungal richness constituting communities, and therefore the main results are driven by dominant endophytes. Thus it cannot be ascertained whether rare OTUs detected locally have a truly restricted distribution, or if such a finding would be due to under-sampling. Such under-representation is common in assessments of microbial diversity and has only been tackled by reaching a considerable sampling depth in species-poor habitats (Taylor *et al*., 2014). Second, the definition of fungal OTUs based on ITS similarity might not be sufficient to resolve closely related species (even when using stringent clustering parameters), and thus might mask hidden biogeographic patterns. Queloz and colleagues (2011) could not detect a biogeographic

pattern in an assembly of root endophytes distributed globally, after applying several molecular markers. However, based on observations in other eukaryotic microorganisms (including fungi; Taylor *et al*., 2006; Gazis *et al*., 2011; Ryšánek *et al*., 2015), and given the broad taxonomic diversity uncovered in our study, we do not discard the possibility of cryptic patterns in the distribution of some fungal groups that showed a cosmopolitan occurrence.

Environmental descriptors of community structure

Endophytic communities were clearly structured along a latitudinal gradient. Latitude gathers a set of co-varying historical, abiotic and biotic gradients that have a strong influence on the distribution of all sorts of organisms (Hillebrand, 2004; Mittelbach *et al*., 2007), including soil and plant-associated fungi (Hoffman and Arnold, 2008; Herrera *et al*., 2010; Tedersoo *et al*., 2012; 2014; U'Ren *et al*., 2012). In our study, latitude determined community composition but not richness and diversity, possibly because the latitudinal range covered was shorter than in other works (Amend *et al*., 2010; Tedersoo *et al*., 2012; 2014). Instead, OTU richness and diversity were negatively correlated with various variables reflecting local precipitation. This situation is similar to what has been found in mycorrhizal fungi (Tchabi *et al*., 2008; Tedersoo *et al*., 2012), but not for above-ground endophytes (U'Ren *et al*., 2012), including those in plants within the Brassicaceae, like *Microthlaspi* (García *et al*., 2013). This pattern contradicts well-known positive effects of rainfall on soil fungal richness (Tedersoo *et al*., 2014, but see Hawkes *et al*., 2011), and hence suggests processes of environmental filtering specific for root symbionts. Water deficiency could increase fungal richness within roots by favouring an active hyphal growth towards roots with a higher water content than the surrounding soil, by compromising host defences against fungal colonization through water stress or by a direct functional modulation of rhizosphere microbial consortia (Van Der Heijden *et al*., 2008; Hawkes *et al*., 2011).

Climatic variables collinear with the latitudinal gradient were the strongest determinants of community composition. These factors were related to annual temperature and precipitation ranges that clearly differentiated endophytic communities from southern areas, with hot and dry summers and wet winters, from those in northern temperate regions, characterized by wetter and colder seasons. Both temperature and rainfall are well-known broad-scale descriptors of fungal occurrence (Arnold and Lutzoni, 2007; Amend *et al*., 2010; Herrera *et al*., 2010; Hawkes *et al*., 2011; Tedersoo *et al*., 2012; 2014; Timling *et al*., 2014). They impose physiological constraints to fungal growth with a differential effect across taxa, affecting growth, spore formation and germination (Torres *et al*., 2003). In addition, the effect of bioclimatic variables on fungal communities might be indirect because they are likely to modulate the structure and productivity of plant communities, and this in turn could affect the microbial diversity associated with particular plants (e.g., Mohamed and Martiny, 2011; Blaalid *et al*., 2012).

Soil characteristics had a negligible influence on endophytic communities at the scale of this study. Soil physicochemistry is a well-known determinant of belowground fungal assemblages, with pH being the factor best explaining large-scale differences (Taylor *et al*., 2014; Tedersoo *et al*., 2014; Timling *et al*., 2014). However, soil features are most likely decisive at local and regional scales, where closely adjacent soil patches can have heterogeneous edaphic conditions. This was shown by Maciá-Vicente and colleagues (2012), who described a profound shift in the structure of root endophytic communities of a single plant species along a gradient of soil salinity of only a few meters. Besides, *Microthlaspi* has specific edaphic preferences that determine only a slight variation in soil characteristics across samples (Koch and Bernhardt, 2004), which are unlikely to exceed the ranges of tolerance for the majority of fungi and thus to represent an important selective factor.

Effect of host phylogeography on endophyte assemblages

Host phylogeny is one of the best descriptors of the plant-associated fungal communities, when widely divergent plant species are considered (Wehner *et al*., 2014). However, the biogeographic structure identified in our study appears to be host independent, which may reflect the relatedness of the plants sampled. While the host genotype had a weak effect in determining whole community structure, it was collinear with other latitude-associated climatic factors that better explained fungal occurrence. Diploid and polyploid *Microthlaspi* species have divergent biogeographic distributions owing to different climate preferences (Ali *et al*., 2015). These explain a somewhat latitudinal distribution of cytotypes (Koch and Bernhardt, 2004), with polyploid *M. perfoliatum* having a wider distribution but preferentially occurring in southern regions, and diploid *M. erraticum* occurring in cooler regions.

Niche occupancy by dominant root endophytes

Our data reveal a clear pattern of distinctive preferences for specific niches or ecological conditions by individual endophytes. Niche occupancy is not only delimited by the distribution of relevant environmental properties, but is also driven by the interaction with competitor species (Silvertown, 2004). In the current study, however, positive

or negative co-occurrences among dominant endophytes seemed to reflect their shared or opposing ecological needs, rather than direct interactions. For example, *Alternaria* sp. OTU002 and *Ilyonectria* sp. OTU005 had latitudinally opposed distributions, which were largely determined by the climate, while their colonies showed a neutral interaction *in vitro*. An alternative explanation for their exclusive presence could be the competition of both groups for the same resources. *Fusarium* sp. OTU001 and *Pyrenochatea* sp. OTU004 had a cosmopolitan occurrence, which was unaffected by environmental or spatial variables. The distribution of *Fusarium* sp. OTU003 was likewise independent from the environment and purely driven by spatial autocorrelation, which defined two apparent foci of occurrence. Interestingly, *Cadophora* sp. OTU006 was the only endophyte the distribution of which was largely determined by soil conditions, showing a negative interaction with pH. Dark septate endophytes within the Helotiales – to which *Cadophora* belongs – are often associated with acidic soils (Sieber and Grünig, 2013), and a recent work found several accessions phylogenetically similar to OTU006 to be strongly correlated with soil properties, including pH (Taylor *et al*., 2014).

Taxonomic identity of Microthlaspi *endophytes*

The largest proportion of root endophytes in *Microthlaspi* belonged to the phylum Ascomycota, consistent with findings for most plants (except for ecto-mycorrhizal trees) based on both cultivation-based and molecular approaches (Sieber, 2002; Porras-Alfaro *et al*., 2008; Herrera *et al*., 2010; Maciá-Vicente *et al*., 2012; Pecoraro *et al*., 2012; Obase and Matsuda, 2014; Wehner *et al*., 2014). The dominance in diversity and frequency of Pleosporales and Hypocreales, the latter with a high proportion of Fusaria, also reflects common patterns of fungal occurrence in roots (Maciá-Vicente *et al*., 2008a; 2012; Márquez *et al*., 2010). Both constitute species-rich orders containing functionally versatile species adapted to a variety of habitats, and their relative presence appears to be modulated by the environment (Maciá-Vicente *et al*., 2008a; Porras-Alfaro *et al*., 2008). Because the occurrence of hypocrealean endophytes was correlated with overall root colonization, it is possible that cultivation methods positively bias towards them, since they often have fast growth rates and easily overgrow other fungi in the isolation plates. Alternatively, this could indicate a systemic colonization of roots by these fungi, which would possibly explain their growth from most of the root pieces plated. The order Helotiales, being the third-most frequent order, contains instances of dark septate root endophytes that predominate in woody hosts in temperate and boreal regions (Sieber, 2002; Sieber and Grünig, 2013).

Several of the frequent OTUs found here overlap with those in a previous description of the cultivable root mycobiota of *Microthlaspi*, from specimens collected in Germany 1 year prior to our sampling (Keim *et al*., 2014). This included species of *Fusarium*, *Ilyonectria*, *Alternaria*, *Pyrenochaeta* and multiple others related to strains identified in our sampling, suggesting a temporal stability of the fungal communities associated with this plant. Remarkably, a large proportion of the endophytic diversity that can be found in healthy wild plants is from genera containing known plant pathogens, many of which are of economic importance in crops. Our work complements previous studies that disclose a cryptic biology of fungi traditionally considered as *bona fide* pathogens because they were first described from diseased plants or are prevalent in agricultural systems (Malcolm *et al*., 2013). This could hint at a switch to pathogenicity because of the highly artificial environment created by intensive forms of agriculture. Besides, it also highlights the problem of oversimplifying the functional roles of root-associated fungi, which is frequent in ecological studies (Aguilar-Trigueros *et al*., 2014) and might lead to the erroneous interpretations of the participation of the fungal biodiversity in the functioning of ecosystems.

Conclusions

Understanding the distribution patterns of fungal root endophytes will help infer the potential functions they play in natural ecosystems, which are as yet largely cryptic. This information will be essential for the long-term monitoring of the global fungal biodiversity, especially in the context of current environmental threats. Here, we show that the distribution of fungal endophytes in roots of an annual plant is determined by the local environment at a continental scale. Geographic distance was a poor descriptor of community structure, suggesting efficient mechanisms for dispersion in this group of fungi. The large-scale changes are principally driven by climatic factors that define a latitudinal gradient of community structure, while soil conditions and host factors appear to have little or a locally restricted effect. Our results also demonstrate particular ecological preferences by individual groups of endophytes, suggesting that they play different functional roles in the ecosystems. To date, there is a limited number of studies on the biogeography of non-mycorrhizal root endophytes. Additional studies based on cultivation-free molecular approaches are ongoing and will provide a more comprehensive view of the spatial scaling of the endophytic fungal diversity. Lastly, the availability of an extensive collection of endophyte strains will warrant the performance of laboratory ecological studies that will help draw a link between their distribution and their potential functional roles.

Experimental procedures

Sample collection

Microthlaspi plants were collected from 52 sites distributed across six European countries (Spain, France, Germany, Croatia, Greece and Bulgaria) and Turkey (Table 1). The samplings were performed in 2013, from mid-April up to early June, roughly corresponding to the flowering period of the plant, and consisted of several field campaigns. Sites were selected according to the presence of an individual *Microthlaspi* population, defined as a cluster of several plant individuals. Populations were separated from one another by a minimum of 2 km. The only exception were populations D-11a and D-11b (Table 1), which grew adjacently but formed clearly different clusters, each with a particular accompanying vegetation. We collected 3–10 healthy-looking and medium-sized plants per population (Table 1), which we carefully uprooted to minimize disruption of roots and stored in cool conditions in food-grade plastic bags until their processing in the laboratory.

Acquisition of environmental and host data

In 42 out of the 52 sites we collected soil samples to characterize the chemical properties of the substrate in which the plants grew. For each site we took multiple soil subsamples from points covering the area of distribution of the plant population, and then pooled them in a single sample. Soils were analysed for pH, conductivity, organic/inorganic carbon and content of macronutrients (N, P, K, S, Na, Mg and Ca; Table S2) by the Soil Science Laboratory Unit of the Goethe University (Frankfurt am Main, Germany). Besides, for all sites we gathered data on elevation and geographic coordinates, which were used to retrieve several bioclimatic variables from the WorldClim (http://www.worldclim.org/; Hijmans *et al*., 2005) and the Consortium for Spatial Information (CGIAR-CSI; Trabucco and Zomer, 2009) data sets. The data set includes 19 variables derived from temperature and precipitation measurements (O'Donnell and Ignizio, 2012), and the degree of aridity (Table S2).

The genotype of the host plants was considered as an additional factor likely to influence the distribution of endophytes. The ITS regions of the ribosomal DNA were sequenced for up to three representative plants of most populations. DNA ploidy levels of these representative plants were determined by flow cytometry calibrated by chromosome counts for reference *Microthlaspi* specimens and ITS sequence comparisons (Ali *et al*., 2015). The estimated ploidy levels were used as a categorical variable in later analyses. Additionally, one representative ITS sequence per population was used to generate a matrix of pairwise genetic distances among populations to include host phylogeny as a numeric variable in statistical analyses. Selection of only one sequence per population was done after assessing a high sequence similarity within populations. One mixed population containing both cytotypes of *Microthlaspi* was excluded from analyses aimed at testing the effect of host on endophytic communities.

Isolation of endophytic fungi from roots

The processing of samples in the laboratory took place in most cases within 72 h after their collection. Roots from every plant were detached and treated individually in every step of the process to isolate endophytic fungi. We chose a mild surface-sterilization protocol for the elimination of microbial epiphytes to avoid over-disinfecting the roots, given their reduced thickness. The protocol consisted of a first wash under running tap water to remove adhered soil particles, and then a surface-sterilization with a 0.5% (v/v) sodium hypochlorite solution for 1 min, followed by three rinses with sterilized deionized water. Roots were then dry-blotted onto sterilized filter paper and cut into *c*. 3-mm long pieces. Ten randomly picked root pieces per plant were plated on a Petri plate containing 0.5% (w/v) Malt Extract Agar (AppliChem, Darmstadt, Germany) supplemented with 0.5 g l^{-1} chloramphenicol to minimize development of bacteria, and with 0.1% (v/v) Triton X-100 (Amresco, Solon, OH, USA) to restrict the spread of fast-growing fungi. We tested the effectiveness of the surface-sterilization protocol by imprinting one third of all root pieces (representing all samples) in the same medium before plating them into the final cultivation plates (Hallmann *et al*., 2006). This yielded fungal growth in 1.7% of the imprints, which we considered acceptable given the large number of root pieces handled and the overall variability in root morphology among individual plants and populations.

The plates with root pieces were incubated at room temperature for a period of 2 months. During this time we recorded the occurrence of fungal colonies as they emerged, and we classified them into morphotypes. We isolated in pure culture one representative colony from each morphotype per plate, yielding a total of 2006 cultures representing 2601 colony counts. All isolates have been deposited in the living fungal cultures collection of IPF hosted at Goethe University, and are available upon request from the authors.

Molecular characterization of strains

We processed 1998 isolates representing all morphotypes for sequencing of their ITS rDNA region. Genomic DNA was extracted from all cultures using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The ITS region was amplified with the fungal-specific primer pair ITS1F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993) in 20 μl of polymerase chain reactions containing 1 μl of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, and 0.5 U Taq polymerase (VWR International, Darmstadt, Germany). Temperature cycles were carried out in a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) and consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension step of 72°C for 5 min. The size of amplicons ranged between 500 and 600 bp in most cases, although some reactions yielded products of up to 1000 bp. The amplified products were sequenced using the same primers by the sequencing laboratory of the Biodiversity and Climate Research Centre (Frankfurt am Main, Germany).

Isolates were assigned to OTUs according to pairwise similarities of ITS sequences, as calculated with the BLASTCLUST tool (ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html) from the NCBI-BLAST package (Altschul *et al*., 1990), using cut-off values that ranged from 90% to 99%. For subsequent analyses, we selected the data set based on the clustering at 97% similarity because it has been shown to provide a good approximation to biological species in studies spanning wide fungal diversities (Taylor *et al*., 2014). Besides, in our case this clustering matched well individual identification of strains, and downstream analyses with data sets based on 98% and 99% cut-offs yielded similar results. The classification of strains was based on comparisons of all sequences with NCBI GenBank entries using BLAST, and with the curated UNITE database (Kõljalg *et al*., 2013) using the Naïve Bayesian classifier running under MOTHUR v1.34.4 (Wang *et al*., 2007; Schloss *et al*., 2009), with a bootstrap support of 80%. Additionally, we repeated the latter approach with an in-house database with sequence and taxonomic data for all fungal ITS sequences in GenBank identified to species level, formatted for its use in MOTHUR. A consensus taxonomy was built for every OTU by either method considering a within-group sharing of at least 51% of the hits at each taxonomic level. Finally, we combined all taxonomic data for a definitive assignment of OTUs up to genus level. Conflicting assignments between the UNITE and the in-house databases were checked manually against GenBank using BLAST. When this was not conclusive, the lowest taxonomic level at which both databases agreed was selected. The taxonomic assignment for each OTU and the GenBank accessions for all sequences are shown in Table S1.

Dual plate assays

To understand potential in-culture interactions among different fungal groups that could have biased the root isolation results, we performed a dual-plate assay as described in Maciá-Vicente and colleagues (2008b). Briefly, representative cultures of the five most frequent fungal OTUs found were confronted in all pairwise combinations in the same medium used for their isolation. Assays were performed in triplicate, and plates were incubated for 1 month after which we recorded presence/absence of inhibitory interactions between colonies (e.g., formation of inhibition halos).

Data analysis

Fungal diversity. All analyses were carried out in R v3.0.2 (R Core Team, 2013) using relevant packages. To analyse fungal diversity and community data, we mostly relied on the package VEGAN v2.2-1 (Oksanen *et al*., 2015). OTU count records for individual root pieces were first assembled into a data matrix containing group-wise colonization percentages per plant, calculated as in Fröhlich and colleagues (2000). This was used to compute overall and averaged values of OTU frequency, richness and diversity indices and richness estimators (Magurran and McGill, 2011) for each plant population. Statistical support for these comparisons was determined with the Kruskal–Wallis Rank sum test (Hollander *et al*., 2013) at a significance level of 0.05. Potential links among richness and diversity data, frequencies of individual OTUs and ecological factors were explored by the calculation of pairwise correlations with the Spearman's rank statistic and linear regression. Because the sampling design included clusters of sites closely spaced and unevenly separated, our data were sensitive to spatial autocorrelation that could inflate type I error in significance tests and invalidate them (Peres-Neto *et al*., 2006). Therefore, we estimated autocorrelation in all bivariate tests using Moran's *I* (Li *et al*., 2007) and corrected it when present using spatial autoregressive models (Dormann *et al*., 2007).

Community analyses. To compare overall fungal communities across plant populations, we calculated dissimilarities in OTU composition among assemblages using the Horn–Morisita index (Horn, 1966). Prior to this we removed singletons (defined as OTUs occurring in only one plant specimen over the survey), and then square-root-transformed the data to reduce the weight of dominant OTUs in the dissimilarities. The utilization of other distance indices or transformation methods yielded similar downstream results, and hence we considered these parameters appropriate. All environmental variables recorded were fitted to the dissimilarities among samples to investigate potential relationships, and significance of these correlations was tested with PERMANOVA (Anderson, 2001). Distances among samples and their correlation with significant factors were visualized by means of an NMDS. The effect of geographic distance on communities was investigated with a Mantel test and a Mantel correlogram by comparing ecological and geographical distances among sites at different ranges.

Variation partitioning. To determine the contribution of ecological factors as predictors of the endophytic community, we used the variance partitioning method following procedures described in Borcard and colleagues (2011) and Legendre and Legendre (2012). This was used to decompose the variation of OTU assemblages into four independent components gathering climatic, soil, spatial and host factors. The spatial component – accounting for unmeasured processes, either intrinsic to the organisms (e.g. dispersal) or environmental (Peres-Neto and Legendre, 2010) – was obtained by the calculation of Moran's eigenvector maps (MEMs; Dray *et al*., 2006), which represent the multivariate structure of the data at all scales covered by the sampling. MEMs were the resulting ordination axes of a principal coordinate analysis (PCoA) of geographic distances among sites, following a weighted Delaunay triangulation connectivity matrix. The geographic coordinates of sites were also included in the spatial component, because MEMs do not cover linear trends associated with latitude and longitude. On the other hand, we excluded elevation from this component because of its strong collinearity with latitude (Spearman's $\rho = -0.86$, $P < 0.001$). The host component included the categorical variable ploidy, and vectors representing the phylogenetic relationship among populations, obtained as the resulting axes of a PCoA ordination of genetic distances in a manner similar to MEMs (Desdevises *et al*., 2003).

Variation partitioning of the community data relied on constrained redundancy analyses (RDAs). We first transformed the singleton-free community matrix using a Hellinger conversion (Legendre and Gallagher, 2001), and then included it

as response variable. The explanatory matrices included factors that individually explained a significant proportion of the variation of the community data, as determined by a forward selection using the R package PACKFOR v0.0-8. We decided to exclude the soil component from these analyses because it only predicted a marginal proportion of the variation, and it reduced considerably the number of observations due to missing data. To assess potential effects of the number of plants collected at each site on the observed structure of communities, we included it as an additional explanatory variable and reported its contribution to the variance explained by ecological components. After variance partitioning, the significance of the variance fractions explained by each component was assessed using constrained RDA with pseudo-*F* tests.

Distribution of dominant OTUs. A modification of the above procedure was used to determine the contribution of the ecological components to the individual variation of the six most frequent OTUs (Peres-Neto and Legendre, 2010). In this case, RDA is equivalent to multiple linear regression because only one response variable is included. We forward-selected factors individually for each OTU and retained all those selected at least once, which were then used as explanatory variables. We excluded the host component from these analyses because of its poor contribution to the variation, and instead we included soil factors at the expense of reducing the number of observations in the models because we deemed them important in explaining the occurrence of particular fungi. Repetition of these analyses with climate and spatial effects alone yielded similar results for these components (data not shown). Fitted scores for linear models representing significant fractions of the variance were represented as surfaces in distribution maps for each OTU.

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Author contributions

MT and JGMV conceived the study. KG, TA, AKB, SP, XX, MT and JGMV collected plant and soil samples. AÇ helped to coordinate samplings in Turkey. KG, SHK and JGMV isolated and processed fungal endophytes. KG and TA characterized plant genotypes. MT and JGMV contributed material/reagents. KG and JGMV analysed the data. KG and JGMV wrote the manuscript with contributions from the other authors.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sequence accessions, taxonomic information, and isolation results for the OTUs described in this study.
Table S2. Ecological factors used in this study.

Chapter 2: Manuscript under review

Glynou K, Nam B, Thines M, Maciá-Vicente JG. Facultative root-colonizing fungi dominate endophytic assemblages in roots of non-mycorrhizal *Microthlaspi* species.

Facultative root-colonizing fungi dominate endophytic assemblages in roots of non-mycorrhizal *Microthlaspi* species

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Summary

- There is increasing knowledge on the diversity of root-endophytic fungi, but limited information on their lifestyles and dependence on hosts hampers the understanding of their ecological functions. We compared diversity and biogeographic patterns of cultivable and non-cultivable root endophytes to assess whether their occurrence is determined by distinct ecological factors.
- The endophytic diversity in roots of non-mycorrhizal *Microthlaspi* spp. growing across Europe was assessed using high-throughput sequencing (HTS) and compared with a previous dataset based on cultivation of endophytes from the same root samples.
- HTS revealed a large fungal richness undetected by cultivation, but which largely comprised taxa with restricted distributions and/or low representation of sequence reads. Both datasets coincided in a consistent dominance of widespread endophytes within orders Pleosporales, Hypocreales and Helotiales, as well as similar associations of community structure with spatial and environmental conditions. Likewise, distributions of particular endophytes inferred by HTS agreed with cultivation data in suggesting individual ecological preferences, and further indicated that the latter are not shared by phylogenetically-related lineages.
- Our findings support that *Microthlaspi* spp. roots are mostly colonized by saprotrophic and likely facultative endophytes, and that processes of competitive exclusion and functional complementarity are important drivers of root-endophytic community assembly.

Keywords: Biogeography, Brassicaceae, Fungal communities, Root endophytes, Saprotrophy, Symbiosis

Introduction

Root microbiomes harbor complex communities of microorganisms that establish interactions both with the host plant and with one another, and which have important effects on plant fitness and diversity (Vandenkoornhuyse *et al.*, 2015). While the role of whole microbiomes on plant-driven processes are becoming evident (Bender *et al.*, 2016), how their functions depend on individual microorganisms and microbe-microbe interactions is barely known (Agler *et al.*, 2016). The ecological functions of plant-associated microorganisms are frequently difficult to assess, but they can be indirectly inferred (to some extent) from their distribution patterns and ecological preferences (Jumpponen *et al.*, 2017), as well as by distinguishing between keystone and subsidiary species in the community (van der Heijden & Hartmann, 2016).

Assessing microorganisms' traits can also be valuable to infer their potential ecological roles (Chagnon *et al.*, 2013) but, because most microbes cannot be cultivated, direct measurements are often difficult. Nevertheless, cultivability itself can be a valuable proxy for important symbiotic functions, since it directly relates with the organisms' dependence on a living host to obtain resources. This is exemplified by arbuscular mycorrhizal fungi, which cannot be grown in pure culture because they entirely depend upon plant photosynthates for growth. On the other hand, ericoid mycorrhizal fungi, which saprotrophically mobilize recalcitrant soil nutrients making them available for their hosts, are easily cultivable (Read *et al.*, 2004). Other common root-colonizing fungi are also cultivable, and are frequent saprotrophs in substrata other than roots, such as soil or plant debris (Rodriguez *et al.*, 2009). Thus far, little is known about the proportion of cultivable vs. uncultivable microorganisms in plant microbiomes (Porras-Alfaro & Bayman, 2011), although recent studies have tackled this question (Oono *et al.*, 2015; Bonito *et al.*, 2016; Siddique *et al.*, 2017). Neither is it known whether the distribution of both groups is differently affected by environmental and geographic factors.

In a recent study, we described the cultivable diversity of endophytic fungi associated with roots of *Microthlaspi* spp. and established its patterns of variation across environmental conditions and space (Glynou *et al.*, 2016). Communities were strongly dominated by Ascomycota, especially in the Pleosporales, Hypocreales and Helotiales, as found by most culture-based studies on root endophytes (Maciá-Vicente *et al.*, 2008,2012; Knapp *et al.*, 2012; Sieber & Grünig, 2013; Keim *et al.*, 2014). Only a few fungi in these orders dominated most communities and were ubiquitous across the host's geographic range, although they

showed variable distributions in association with different environmental and spatial factors (Glynou *et al.*, 2016). Altogether, these findings support a different niche occupancy by distinct fungal lineages, suggesting a complementarity of functions within endophytic communities. But whether these cultivable fungi represent an important fraction of fungal endophytic communities remains uncertain. Whereas *Microthlaspi* spp. is non-mycorrhizal (Regvar *et al.*, 2003), it is possible that other obligate—hence likely uncultivable—symbionts are more important components of its root mycobiome.

Here, we relied on a cultivation-independent approach based on Illumina Miseq high-throughput sequencing (HTS) to evaluate the fungal diversity within roots of *Microthlaspi* spp., using the same samples processed by Glynou and colleagues (2016) for endophyte isolation. HTS has promoted thorough microbial ecology studies and contributed considerably to the identification of fungal diversity and community dynamics (Peay *et al.*, 2016). Compared to cultivation-based approaches, amplicon sequencing achieves deeper sampling, detects uncultivable and slow-growing taxa, and is not subject to overrepresentation of fast-growing saprotrophic groups (Peay *et al.*, 2008; Porras-Alfaro & Bayman, 2011).

Combining biogeographic data from environmental sequencing and cultivation methods can help determine the trophic dependence of fungal root endophytes on hosts, and whether the assembly in endophytic communities of fungi with different symbiotic strategies is differently affected by environmental and geographic factors. A low representation of uncultivable endophytes would imply that communities are dominated by saprotrophs, likely to be facultative root colonizers able to exploit other substrata. However, based on previous results (Bonito *et al.*, 2016; Siddique *et al.*, 2017), we hypothesize a large proportion of uncultivated endophytes, due to either a more thorough diversity assessment achieved by HTS than by cultivation, or to a high presence of fungi with slow growth or complete lack thereof on cultivation media. The latter situation would suggest a higher specialization of endophytic communities toward the symbiotic lifestyle than currently expected from cultivation data. Lastly, evaluation with HTS of the influence of ecological factors and geographic distance on the assembly of endophytic communities, as well as on the distribution of individual species, can confirm or falsify patterns disclosed by cultivation. In this study, we aimed (1) to assess the overall fungal endophytic diversity in roots of *Microthlaspi* spp. using HTS, (2) to evaluate the proportion of cultivable and uncultivable fungal richness by comparing with previous cultivation-based results from Glynou and colleagues (2016), and (3) to assess main

ecological drivers determining community structure and distribution of fungal root endophytes, emphasizing potential differences between cultivable and uncultivable fungi.

Materials and Methods

Sample collection and processing

The plant material used was collected by Glynou and colleagues (2016), and corresponds to root samples from 43 of the 52 *Microthlaspi* spp. populations investigated in that study (Table 1). In brief, *Microthlaspi* spp. plants were collected in spring 2013 from populations across Europe. Roots were surface-sterilized in 0.5% (v/v) sodium hypochlorite for 1 min followed by three rinses with sterile deionized water, and then processed for isolation of fungal endophytes as described by Glynou and colleagues (2016). The surface-sterilization procedure eliminated rhizoplane fungi in more than 98% of the samples (Glynou *et al.*, 2016). The remaining root material was stored at -78^{o}C until processing for DNA extraction. Many root samples were small and no material was left after cultivation. In these cases, additional samples collected from the same *Microthlaspi* spp. populations, but not processed for isolation, were included if available. This resulted in a variable number of plants per population (Table 1), totaling 383 samples.

Geographic, bioclimatic and soil physicochemical data from each plant population were obtained from Glynou and colleagues (2016). Therein, bioclimatic variables were calculated from the WorldClim (Hijmans *et al.*, 2005) and the Consortium for Spatial Information (CGIAR-CSI; Trabucco & Zomer, 2009) datasets. Soil data were obtained from direct measurements from soil samples collected upon samplings, analyzed by the Soil Science Laboratory unit of Goethe University. Host factors related to plant's ploidy (indicative of species affiliation; Ali *et al.*, 2016) were likewise compiled. A detailed description of all factors included is provided in Table S1.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from individual root samples using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. We normalized the amount of

total DNA per sample using a Nanodrop 2000 (NanoDrop products, Wilmington, USA), and then attempted to amplify fungal DNA from individual samples for downstream analyses. However, this resulted in an uneven amplification success (approx. 60% of the samples) due to the small size of roots probably coupled scarce fungal colonization, rather than to the low quality of DNA, as found elsewhere (Maciá-Vicente *et al.*, 2016). Therefore, we decided to pool samples population-wise using 30 ng of DNA from each plant extract, to yield a total of 43 DNA samples. Pooled DNA samples were diluted to a final concentration of 10 ng μl^{-1} and used for a first amplification of fungal rDNA ITS regions using primers ITS1F and ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993). Reactions were performed in 25 l containing 20 ng of DNA template, 0.2 mM dNTPs, 0.5 M of each primer, 0.8 µg μl^{-1} bovine serum albumin, and 0.5 U Phusion High-Fidelity DNA Polymerase (New England Biolabs). Thermal cycles were done in a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany), and consisted of an initial denaturation step of 98°C for 2.5 min followed by 25 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension step of 72°C for 5 min. Amplifications were performed five times and amplicons were pooled, purified using the EZNA Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) and quantified by band intensity in agarose gels. Three barcode PCR amplifications followed, using tagged primers (Table S2) with the aforementioned PCR reagents and thermal conditions, but including only ten amplification cycles. The samples were pooled and quantified by band intensity in an agarose gel.

We included negative controls during the extraction and amplification steps, which were also processed for sequencing to evaluate potential contaminations along the entire process. In order to evaluate variations in the amplification and sequencing efficiency across different fungal lineages, as well as to calibrate operational taxonomic unit (OTU) delimitations, we generated amplicons from a mock fungal community. It was achieved by pooling equimolar DNA amounts from representative isolates from the 20 most abundant OTUs obtained by Glynou and colleagues (2016). In addition, to further test OTU delimitation, we added six individual sequencing reactions from DNA of representatives of each of the six most frequent OTUs. The latter revealed a contamination in the amplification of the second-most abundant group (*Alternaria* sp. OTU002 in Glynou *et al.* 2016), and hence this sample was omitted from analyses of the controls.

All amplified and tagged DNA samples were finally pooled by adding 100 ng of DNA per sample, and the pooled sample was then purified using the Zymoclean™ Gel DNA Recovery

Kit (Zymo Research, Freiburg, Germany) and quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Pooled amplicons were sequenced by Eurofins Genomics GmbH (Ebersberg, Germany) using the Illumina MiSeq platform.

Sequence processing

A first quality check of raw sequences using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) revealed a much higher quality of R1 than R2 reads, and a low quality of the 5.8S region that hindered proper assembly of reads (Fig. S1). The same outcome has been found and discussed elsewhere in datasets obtained using the ITS1F/4 primer pair, owing to amplicon length (Siddique & Unterseher, 2016; Eusemann *et al.*, 2016; Unterseher *et al.*, 2016). Hence, we decided to follow the approach of data analysis therein published, based on the R1 dataset only.

The detailed pipeline for the quality filtering of sequences, their clustering into OTUs and their annotation is available online (https://figshare.com/s/05fed049a34233b0e84e). In brief, sequences were first demultiplexed and then quality-filtered by retaining only those with at least 75% of their length with phred scores 30. The full and partial ITS1 regions were extracted using ITSx v1.0.11 (Bengtsson-Palme *et al.*, 2013) running in parallel sessions in the FUCHS computer cluster of the Center for Scientific Computing of Goethe University. We used tools in the USEARCH pipeline (Edgar, 2010) for subsequent filtering and clustering steps. Sequences were truncated to a length of 133 bases to avoid the effect of variable sequence lengths in similarity clustering. Non-singleton sequences were clustered into OTUs using different sequence similarity thresholds (97–100%). Potentially chimeric sequences were discarded by comparisons with the reference database provided by UNITE (Nilsson *et al.*, 2015). A final set of high-quality sequences representing individual OTUs at a 97% similarity level were used to assemble a contingency table of OTUs per plant population from the raw sequence file. Sequence data has been deposited in the NCBI Sequence Read Archive under accession SRP106137.

Sequence annotation

We conducted a comprehensive procedure of sequence annotation to maximize information on the phylogenetic placement of OTUs and discard artifacts. We first used the naïve

bayesian classifier (Wang *et al.*, 2007) as implemented in MOTHUR v1.34.4 (Schloss *et al.*, 2009) to compare representative OTU sequences against the UNITE database of reference fungal ITS sequences (Kõljalg *et al.*, 2005), retaining only annotations with a bootstrap support 60%. This procedure was not sufficient to identify many OTUs at more inclusive taxonomic levels, and several groups were constituted by potential sequence artifacts. Therefore, we applied an additional annotation step by conducting phylogenetic analyses of all representative OTU sequences with an in-house automated pipeline. For each sequence, the pipeline performs BLAST comparisons (Altschul *et al.*, 1990) against the full NCBI GenBank database, and against subsets of it containing only sequences from vouchered strains or species type specimens, retaining a maximum of 10 best hits from each dataset. A maximum likelihood (ML) phylogenetic tree is then built with the retrieved sequences using RAxML v8.0.0 (Stamatakis, 2014) with a GTRGAMMA model and 100 bootstrap replications. We manually examined all sequences that failed to deliver a phylogenetic tree and discarded most of them as likely artifacts. Finally, we used phylogenies to manually annotate OTUs that remained unidentified above the order level after the bayesian classification, when possible. OTU annotations are provided in Table S3, and all phylogenetic trees are available online at https://figshare.com/s/05fed049a34233b0e84e.

In order to identify OTUs that had been previously detected via cultivation, we compared with BLAST all representative sequences against a local database built with sequences obtained by Glynou and colleagues (2016), corresponding to fungi isolated from the 43 plant populations studied here. A second set of comparisons was done to match sequences obtained from each population with only those derived from isolates obtained from the same plant population. We considered that two sequences belonged to the same OTU when coverage and identity of BLAST alignments were above 95% and 97%, respectively. To evaluate the proportion of matched sequences, we compared the BLAST statistics with those involving comparisons with the full GenBank and UNITE databases (Fig. S2).

Statistical analyses

Statistical analyses were carried out in R v3.3.1 (R Core Team, 2016) with use of relevant packages. The R script and input datasets are available online at https://figshare.com/s/05fed049a34233b0e84e. We first removed from the dataset OTUs exclusive of the negative controls and those represented globally by less than five reads. The

resulting quality-filtered dataset was used for the assessment of richness and diversity. However, an alternative assessment was performed using a pruned dataset, in which single reads per cell of the contingency table, which can arguably be considered noisy detections, were discarded. The pruned dataset was used in the final downstream diversity and community structure analyses, because it showed a full coverage of diversity.

Fungal richness, diversity and read abundances were calculated in the overall dataset and per population, by applying common diversity indices and rarefaction curves using tools in the R package VEGAN v2.3-5 (Oksanen *et al.*, 2015). Diversity was calculated using Shannon and Simpson indices, and then transformed to effective species numbers (Hill numbers 1 and 2, respectively; Jost, 2006). Assessments of richness and diversity variation across samples or in relation with ecological factors were done by means of linear regression analyses, after logarithmic or square-root transformation of strongly skewed variables. In all cases, regressions were performed with partial residuals after subtracting the effect of the number of reads. The number of reads per sample was somewhat collinear with the number of plant samples per population (Pearson's r=-0.35, P=0.02). While the former was strongly correlated with richness per sample (r=0.66, P<0.001), the number of plants sampled had no strong association with these values (r=-0.03, P=0.87). Hence, correcting for the number of reads in regressions was considered sufficient for controlling the effects of the asymmetric sampling.

Community composition analyses were performed using a Hellinger-standardized dataset (Legendre & Gallagher, 2001). Relationships in OTU composition among samples were investigated via calculation of Bray-Curtis dissimilarities, and visualized by means of non-metric multidimensional scaling (NMDS). Correlations of fungal community structure with geographic, bioclimatic, soil and host factors (host ploidy/species) were explored permutational analysis of variance with function *adonis* of VEGAN (Anderson, 2001). The analyses were repeated using the Jaccard's incidence-based dissimilarity index, to evaluate the effect on results of using abundance or incidence data. Spatial factors, explaining potential non-random patterns in the data not accounted for by the measured environmental factors (Peres-Neto & Legendre, 2010), were also included as principal coordinates of neighbor matrices (PCNM) vectors (Legendre & Legendre, 2012). PCNMs were calculated from geographic coordinates of the sampling sites using function *pcnm* of VEGAN, and those with a significant relationship with variation in community similarity were forward-selected using package PACKFOR v0.0-8 (Dray *et al.*, 2009). The relative effect of categories of ecological factors (e.g. climatic, soil and spatial variables) was calculated by variation partitioning

(Legendre & Legendre, 2012). Lastly, we calculated the effect of geographic distance on community structure for the core and full OTU datasets, using Mantel test and Mantel correlograms (Legendre & Legendre, 2012), and by comparing the range sizes of distribution for each out. The latter were calculated as the maximum distance between the sampling sites in which each OTU was found (Cox *et al.*, 2016). Differences in range sizes between cultivated and uncultivated OTUs were visualized with a boxplot, and tested for significance using Wilcoxon's rank sum test.

To identify the effects of environmental and spatial variables on individual OTUs, we focused on a subset of the data including only OTUs represented in at least 30% of the populations (core community, onwards; Bálint *et al.*, 2015). We relied on tools in package MVABUND v3.11.9 (Wang *et al.*, 2012) to build multispecies generalized linear models (GLMs). The method simultaneously fits GLM models of individual OTU abundances to selected ecological variables and then generates global estimates for the full dataset (Warton *et al.*, 2015). In order to restrict the number of explanatory variables in these analyses, we used function *best.r.sq* to forward select the three most explanatory factors among each of the climatic, soil and spatial categories. The selected variables were then used to model OTU occurrences in the core mycobiome with function *traitglm*. We extracted from these models the interaction coefficients indicating whether occurrence of each OTU was positively or negatively associated with any of the ecological factors tested. The run was set to apply LASSO penalties, which simplify interpretation of results because they set to zero interaction coefficients not contributing significantly to results upon model selection (Warton *et al.*, 2015).

We investigated if ecological preferences in OTU distributions were conserved among phylogenetically close lineages within the Pleosporales, Helotiales and Hypocreales, the three dominant orders of root endophytes. We used representative sequences from core OTUs in each group to build three ML phylogenies, as explained above. Phylogenetic conservatism in the multispecies GLMs interaction coefficients for each ecological factor was estimated with the *K* statistic (Blomberg *et al.*, 2003), using function *phylosig* of R package PHYTOOLS v0.5 (Revell, 2012). *K* close or above 1 indicates phylogenetic signal, whereas $K=0$ indicates absence of phylogenetic signal.

Results

Description of the sequencing dataset

A total of 2,071,320 reads were retained after demultiplexing, quality filtering, ITS1 extraction and chimera-filtering of sequences. A total of 1,212 OTUs were defined based on 97% sequence similarity from these reads, after having assessed their quality via BLAST searches and phylogenetic analyses. Removal of reads in negative controls as well as rare OTUs with less than five total reads resulted in a final 2,070,711 reads, grouped in 1,003 OTUs in the root samples from the 43 *Microthlaspi* spp. populations. An additional 4,078 reads were removed after pruning single reads from the dataset, which lowered the number of OTUs to 995. The number of reads per sample ranged from 7,382 to 95,363, with a mean of 48,061 and a median of 48,363 (Fig. S3a), and showed a skewed distribution with a large proportion of very rare OTUs (Fig. S3b). Sequencing of a mock community with equimolar amounts of genomic DNA from different fungi detected all OTUs, although one (OTU2123, corresponding to OTU020 in Glynou and colleagues, 2016) was only represented by 2 reads (Fig. S4). The number of reads per OTU was highly variable, ranging from 173 to 12,040 reads (Fig. S4), with a median of 3,408 reads. Amplification of five individual OTUs showed a rather homogenous grouping, indicating low intra-fungus variability (Fig. S5).

The rarefaction curves showed that our sampling covered nearly all the diversity of root-endophytic fungi in *Microthlaspi* spp., either when considering the full or the pruned dataset (Fig. S6a). Calculation of curves per plant population showed a lack of saturation in the full dataset (Fig. S6b), while saturation was achieved in nearly all cases when the pruned dataset was considered (Fig. S6c).

Sequence annotation and comparison with cultivation data

We obtained an average of 187±42 (SD) OTUs per population, ranging from 109 to 272 OTUs per site (Table 1). The numbers of effective species per sample had averages of 14.2±7.7 and 7±3.9 according to Hill's numbers 1 (Shannon) and 2 (Simpson), respectively (Table 1). We compared the values of richness and read numbers across samples with those obtained by cultivation, using linear regressions. The numbers of HTS reads were strongly correlated with colonization percentages, representing the proportion of root fragments yielding isolates in culture (Fig. 1a; slope=545.8±183.4, adj. R^2=0.16, F_{41}=8.9, P=0.005). Likewise, richness values obtained using both methods were positively correlated after correcting for sampling depth (Fig. 1b; slope=23.6±5.2, adj. R^2=0.32, F_{40}=20.3, P<0.001).

Of the 995 OTUs in the HTS dataset, 251 (25.2%) had been detected by cultivation in at least one plant population (Fig. 1c), whereas the large majority could only be assigned to other fungal records in the UNITE and NCBI GenBank databases (Fig. S2). Contrastingly, the percentage of overlapping OTUs per sample detected by either method decreased to an average of 12.7±3.9, with a resulting high proportion of uncultivated groups (Fig. 1c). Overall, 45 OTUs isolated from *Microthalspi* spp. roots could not be detected by HTS, with values across samples ranging from 0 to 12 OTUs (Fig. 1c). The proportion of cultivable OTUs per sample was negatively correlated with several variables of precipitation, and positively with mean temperature of the wettest quarter and soil available potassium (Fig. S7).

The largest proportion of OTUs in our HTS dataset was assigned to phylum Ascomycota (81.1%) and then to Basidiomycota (15.2%), which were followed by a minor representation of other groups (Fig. 2). At the order level, Pleosporales, Helotiales and Hypocreales represented the largest proportion of OTUs, while a large number of groups remained unclassified. These patterns resembled those obtained by cultivation, although the latter showed lower values of helotialean and unidentified OTUs (Fig. 2). At the OTU level, the dominant fungi found by Glynou and colleagues (2016) appeared to be also among the most frequent OTUs as detected by HTS (Table 2). Two OTUs in this selection (OTU7 and OTU14) matched the same isolate OTU.

Relationship between ecological factors and community structure

We inspected the relationship between OTU richness and ecological factors using linear regression analyses, in which the unequal amount of reads per sample were accounted for. Variation in richness was not associated with the latitudinal gradient, which gathers a large variation of many collinear ecological factors (Fig. 3a). Instead, it was significantly and negatively correlated with several temperature predictors (Figs. 3b, S8), and to a lesser extent with precipitation factors (Fig. S8).

An NMDS ordination based on Bray-Curtis dissimilarities (stress=25%) showed a clear structure of communities along latitude, alongside several collinear bioclimatic, soil and spatial variables (Fig. 4a; Table S4). Similar results were obtained using the Jaccard's incidence-based index in the ordination (Fig. S9). Climatic, soil and spatial factors explained a 10% of the overall variation of community similarity (Fig. 4b). Spatial factors contributed the most to total variation, although the three variable categories were largely collinear.

Geographic distance was correlated with overall community similarity (R=0.18, P<0.001). The Mantel correlogram showed that communities from sampling sites closer than 50 km had a significantly greater resemblance than more distant sites (R=0.31, P<0.001; Fig. 4c). The assessment of the range sizes of individual OTUs showed a large proportion of endemic groups with a restricted occurrence up to 200 Km (Fig. 4d). However, many OTUs had a widespread distribution that spanned nearly all the geographic range sampled (Fig. 4d). We found a link between culturability of OTUs and their range sizes (W=784970, P<0.001), in which cultivable OTUs tended to have broader ranges than uncultivated OTUs. However, the latter presented a wide variability in their distribution ranges (Fig. 4e).

Ecological preferences of individual OTUs

We focused on the core 183 OTUs occurring in at least 30% of the plant populations to explore their individual associations with ecological factors, as well as potential phylogenetically conserved habitat preferences. A first forward selection allowed to identify variables from climatic, soil and spatial categories which had the strongest association with OTU occurrences (Fig. 5). Additionally, we included latitude and longitude as spatial factors not accounted for by PCNMs (Fig. 5). The sign and magnitude of coefficients varied highly across OTUs, indicating either positive or negative correlations with individual factors (Fig. 5). However, they did not follow evident patterns across related lineages, even when a certain degree of phylogenetic conservatism was found (Fig. 5 and Table S5).

Discussion

The assessment of root-endophytic fungal communities of *Microthlaspi* spp. using a cultivation-free approach uncovered a large diversity of fungi that had gone undetected by cultivation of the same root samples (Glynou *et al.*, 2016). Nevertheless, most OTUs that appeared to dominate communities according to sequence reads also represented an important component of the cultivable diversity, both in terms of incidence and abundance (Glynou *et al.*, 2016). The overlap in the detection of these groups, together with the frequent affiliation of OTUs to taxa eminently composed of saprotrophs—e.g. Pleosporales, Helotiales and Hypocreales—suggest that facultative endophytes predominate in these communities. Conversely, arbuscular mycorrhizal fungi were absent, in contrast to what is found in plant

species that grow in habitats subject to nutrient limitation (Soudzilovskaia *et al.*, 2015). This is hardly surprising given the documented lack of classical mycorrhizal associations within Brassicaceae (Fitter, 2005). However, other widespread root biotrophs such as the Sebacinales (Weiß *et al.*, 2016) also were remarkably underrepresented, despite being able to establish stable interactions with Brassicaceae species (Lahrmann *et al.*, 2015; Banhara *et al.*, 2015).

Many of the dominant fungi in our dataset are related to common facultative pathogens of Brassicaceae and other plants (Thomma, 2003; Michielse & Rep, 2009; Travadon *et al.*, 2015). Nevertheless, it is unlikely that most endophytes function as pathogens in nature, given their ubiquity in healthy hosts and their ability to become mutualists under certain conditions (Rodriguez *et al.*, 2009; Hiruma *et al.*, 2016; Fesel & Zuccaro, 2016). Different fungal groups probably adopt context-dependent strategies resulting from the interplay among host identity, environment, and microbial interactions (Mandyam & Jumpponen, 2015). In any case, fungi in the Pleosporales (e.g. *Alternaria* and *Pyrenochaeta* spp.), Hypocreales (*Fusarium* and *Ilyonectria* spp.) and Helotiales (*Cadophora* spp.) are dominant root endophytes of *Microthlaspi* spp. (Keim *et al.*, 2014; Glynou *et al.*, 2016) and other plants (Maciá-Vicente *et al.*, 2008; Knapp *et al.*, 2012; Bonito *et al.*, 2016), and they are found both by sequencing and cultivation. Hence, these groups seem important components of natural root-endophytic communities, in which they may play relevant functions. Members of these lineages have distinctive sets of life history and functional traits suggestive of complementary niche occupancies, which may favor their frequent co-occurrence in roots (Kia *et al.*, 2017).

General diversity patterns

Our sequencing approach appeared to uncover the full OTU richness in the root-endophytic mycobiome of *Microthlaspi* spp., even though we cannot discount that some taxa went missing given the unequal detections yields in our mock community, and known biases of PCR primers (Bellemain *et al.*, 2010; Ihrmark *et al.*, 2012). Differently to overall OTU accumulation curves, those for individual plant populations often failed to reach a plateau, indicating that OTUs missing in these samples were detected elsewhere in our sampling. Pruning single values in the dataset resulted in a sharp saturation of population-wise accumulation curves, proving that these single reads strongly condition the apparently unobserved diversity. HTS is inherently noisy, so that many reads and OTUs are discarded upon data filtering (Lindahl *et al.*, 2013). Thus, such single reads (0.002% of the already

quality-filtered reads) can be feasibly considered artifactual detections. While their removal has little effect on diversity patterns and community structure results (Gobet *et al.*, 2010; Lindahl *et al.*, 2013), they markedly affect absolute measures of richness and the interpretation of sampling coverage, the latter ranging from an insufficient to a complete description of individual communities. We considered the latter situation the most probable, although we cannot completely discard the possibility of extremely diverse communities suggested by the unpruned dataset.

Comparison of sequencing and cultivation data

Our dataset provides a valuable resource to compare the performance of cultivation-based and cultivation-free methods in assessing fungal diversity. The match between results from both methods is a debated topic (Porras-Alfaro & Bayman, 2011). Our result agree with recent studies (Bonito *et al.*, 2016; Siddique *et al.*, 2017) in describing a core set of endophytes detectable via both methods likely representing the dominant taxa, plus a large diversity of OTUs exclusively detected by direct sequencing. It can be argued that the latter represent fungi with particular nutritional requirements or low saprotrophic potential, such as biotrophs or fungi linked to niches constructed by other microbes (Buser *et al.*, 2014). However, cultivation approaches rarely achieve complete descriptions of fungal richness in natural communities, and this is true for the study of Glynou and colleagues (2016). Hence, a fraction of the uncultivated OTUs may correspond to cultivable but rare or slow-growing fungi, which could account for 14–47.4% of the total diversity depending on the richness estimator considered (Glynou *et al.*, 2016). These could be fastidious species or the result of inconsistencies in OTU delimitation or BLAST comparisons with sequences from isolates. The same may be partly applicable to fungi exclusively found by cultivation, which alternatively might correspond to fungi with a restricted colonization of certain root portions that were exclusively processed for platting on agar media. By applying the above ratios of undetected cultivable OTUs, we can estimate the proportion of purely uncultivable OTUs in our HTS dataset in the range of 487 (49%) to 683 (69%) OTUs. Our dataset contains fungal lineages known to be uncultivable, such as the Rozellomycota (*syn.* Cryptomycota; Jones *et al.*, 2011; Corsaro *et al.*, 2014) or many Agaricales (Hibbett *et al.*, 2016). Their overall small representation in OTU numbers, however, is discordant with the estimated percentages of uncultivable fungi, hence we consider these as tentative figures in need of further evaluation.

The patterns of overall fungal abundance and richness in *Microthlaspi* spp. roots were correlated with those obtained by cultivation, indicating a reproducible assessment of both diversity measures. This validation of both methodologies is relevant because they have frequently and independently been applied to study fungal biodiversity patterns. In spite of this relative concordance in overall fungal detection, read abundances for individual OTUs represented by equimolar DNA amounts in the mock community showed a high variability that is in agreement with previous studies (Amend *et al.*, 2010; Ihrmark *et al.*, 2012; Nguyen *et al.*, 2015). The differential signal across fungi can result from differences in nuclei numbers or ITS copies per biomass unit, heterogeneous PCR amplification efficiencies, or biases during sequence processing. Cultivation also introduces multiple biases in fungal quantification (Schulz & Boyle, 2005), thus it is unfeasible to obtain a complete picture of the real root colonization by individual endophytes in community-wise studies. However, both HTS and cultivation coincided in most of the dominant fungi, which hints to their actual large representation in the fungal assemblages targeted by our study.

Ecological drivers of fungal endophytic community assembly

Root-endophytic diversity did vary along gradients of climatic variables independent of latitude, while the latter defined a clear structure in the composition of communities. These results agree with the patterns described by Glynou and colleagues (2016), although the contribution of particular environmental variables to overall community variation differs. Mainly, we observed a stronger association of assemblages with soil characteristics and spatial effects than detected by cultivation (Glynou *et al.*, 2016). Our results indicate that most cultivable fungi have potentially wide niche breadths and cosmopolitan distributions (Glynou *et al.*, 2017), with occurrences likely associated with range-wide variables like climate. Conversely, HTS revealed an important proportion of previously overlooked OTUs, endemic of restricted localities and that probably are sensitive to local shifts in their habitats (Poisot *et al.*, 2011). Soil characteristics are highly variable at a local scale, where heterogeneous edaphic conditions can vary across adjacent soil patches. Likewise, variables lumped in the spatial effect are likely to be locally structured if they describe phenomena like autocorrelation in species' distribution due to dispersal limitation, in accordance with the patchy structure of communities unraveled by Mantel correlograms. These might be associated with the high endemicity of uncultivable OTUs.

Niche partitioning in the root mycobiome

Our findings suggest a large variability of ecological preferences across individual root-endophytic fungi. This is consistent with previous results from soil fungal communities (Taylor *et al.*, 2014), and support a strong niche partitioning in root endophytic communities. This observation is reinforced by the variable morphological, physiological and functional traits of phylogenetically distant endophytic fungi that commonly co-exist in *Microthlaspi* spp. roots (Kia *et al.*, 2017). Inter-species complementarity reduces competition, thereby promoting co-occurrence in communities (Maherali & Klironomos, 2007). Moreover, the ecological preferences across OTUs were not phylogenetically conserved, since close lineages often presented distinctive occurrence patterns. The differential niche occupancy by close endophytes may result in a functional stability of root-associated communities. Because genetically related fungi likely play similar ecological roles within assemblages, their distinctive responses to environmental conditions can lead to a functional stability of endophytic communities, as loss of functions with species turn-over across environmental gradients is prevented (Maherali & Klironomos, 2007).

Conclusions

Our findings suggest that the root-endophytic mycobiota of *Microthlaspi* spp. is dominated by facultative endophytes, with saprotrophic lifestyles and widespread distributions. They also indicate that the frequency of non-cultivable, potentially biotrophic endophytes is high, but their distribution is restricted locally. It is conceivable that the structure and function of endophytic communities in *Microthlaspi* spp. are more associated with fungal pathogens developing context-dependent, asymptomatic interactions, than with mutualistic biotrophs. We provide evidence that the occurrence of the dominant endophytes is affected by the local environment, and that the communities they form are shaped by processes of competitive exclusion and niche partitioning. The implementation of network analyses could give an insight on the co-occurrence patterns among OTUs, while trait-based analyses could resolve the effect of trait complementarity on the organization of communities, and on the outcome of interactions with plants. Finally, it is important to study the ecology of the uncultivable taxa which, despite their rarity, may contribute significantly to community assembly and to important functions relevant for the symbiosis with the host (Jousset *et al.*, 2017).

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Author Contributions

KG, MT and JGMV designed the research. KG and BN conducted the research. MT and JGMV contributed reagents and analytical tools. KG and JGMV analyzed the data and drafted the manuscript. All authors contributed to the manuscript.

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Table 1. Description of sampling sites, and results of fungal abundance and diversity.

Country	Site	Coordinates	Elevation (m a.s.l.)	Host[a]	n[b]	Reads	Diversity (Hill numbers)		
							S (^0D)	H' (^1D)	$1/D$ (^2D)
Bulgaria	BG-007	42.50 N / 22.82 E	614	*M. perfoliatum*	6	91618	226	6.6	2.6
	BG-010	42.70 N / 22.83 E	770	*M. erraticum*	9	85200	239	10.2	4.1
	BG-011	42.67 N / 22.84 E	740	mixed	3	41885	182	16.7	7
	BG-012	42.66 N / 22.81 E	773	*M. perfoliatum*	7	28369	172	22.4	13.8
	BG-013	42.63 N / 22.73 E	837	*M. perfoliatum*	9	41273	182	23.8	13
	BG-014	42.59 N / 22.72 E	711	*M. erraticum*	2	30283	151	13.2	9.5
	BG-015	42.57 N / 22.69 E	685	*M. erraticum*	9	32376	202	31.9	15.4
	BG-023	42.91 N / 22.83 E	621	*M. perfoliatum*	5	70788	215	11.1	5
Germany	D-100	49.54 N / 09.34 E	415	*M. perfoliatum*	21	58454	272	25.1	12.2
	D-101	49.68 N / 10.00 E	278	*M. erraticum*	14	62257	226	11.9	4.7
	D-102	49.45 N / 09.82 E	281	*M. erraticum*	10	10963	150	18.9	8.3
	D-103	49.27 N / 09.84 E	299	*M. erraticum*	13	27609	183	25.6	12
	D-104	48.61 N / 09.53 E	515	*M. erraticum*	14	7382	110	14.8	6.3
	D-105	48.55 N / 10.12 E	481	*M. erraticum*	13	14091	165	24.7	12
	D-11a	50.37 N / 07.22 E	504	*M. erraticum*	4	46451	187	27	13.8
	D-11b	50.37 N / 07.22 E	504	*M. erraticum*	10	50275	172	3.8	1.7
Spain	ES-001	38.04 N / 02.48 W	1630	*M. perfoliatum*	14	67723	179	15.2	8
	ES-002	38.05 N / 02.54 W	1612	*M. perfoliatum*	12	23568	150	13.6	6.1
	ES-003	38.09 N / 02.56 W	1253	*Microthlaspi* sp.	12	29501	156	8.3	4.4
	ES-004	37.97 N / 02.45 W	1204	*M. perfoliatum*	12	53850	185	9	5.4
	ES-005	37.14 N / 03.48 W	1351	*M. perfoliatum*	13	19649	133	12.3	5.4
	ES-006	37.13 N / 03.43 W	1669	*M. perfoliatum*	10	48596	194	13.9	8

	ES-010	42.81 N / 04.25 W	1055	*M. perfoliatum*	9	40677	222	33.1	16.6
	ES-012	42.87 N / 04.15 W	1305	*M. perfoliatum*	9	65343	242	15.4	6.9
France	F-007	47.11 N / 06.07 E	543	*M. erraticum*	8	30360	179	16.6	5.8
	F-008	47.08 N / 06.07 E	533	*M. erraticum*	9	32445	183	15.5	7.6
	F-009	47.18 N / 05.46 E	216	*M. perfoliatum*	8	79561	242	9.7	5.2
	F-010	47.20 N / 05.43 E	198	*M. erraticum*	9	53839	249	9	2.7
	F-011	47.32 N / 04.60 E	446	*M. perfoliatum*	2	88904	218	11.9	5.9
	F-013	47.03 N / 03.59 E	215	*Microthlaspi* sp.	3	72239	196	3.5	1.9
	F-014	47.19 N / 01.20 E	121	*M. perfoliatum*	6	49093	213	19	11
	F-015	46.41 N / 00.22 E	112	*M. perfoliatum*	9	56728	192	6.2	2.5
	F-021	44.58 N / 05.38 E	1260	*M. perfoliatum*	6	81978	236	5.3	2.1
	F-024	44.50 N / 05.42 E	734	*M. erraticum*	4	56151	207	12.9	6.1
Greece	GR-001	39.81 N / 20.77 E	1065	*M. perfoliatum*	11	51120	237	14.4	7.2
	GR-002	38.94 N / 21.76 E	1410	*M. perfoliatum*	10	73157	253	17	8.4
	GR-003	38.91 N / 21.74 E	1283	*M. perfoliatum*	18	24968	119	7.3	4.3
	GR-004	38.91 N / 21.83 E	905	*M. perfoliatum*	8	31834	163	22.2	10.8
	HR-021	44.16 N / 15.58 E	795	*M. erraticum*	6	43912	116	6.6	4.4
	HR-022	44.19 N / 15.52 E	574	*M. erraticum*	3	95363	154	3.8	2.8
	HR-023	44.24 N / 15.54 E	760	*M. perfoliatum*	3	48363	146	4.4	2.8
	HR-025	44.46 N / 15.40 E	755	*M. erraticum*	12	31776	134	7.3	4.8
	HR-028	44.59 N / 15.44 E	525	*M. erraticum*	8	16661	109	8.1	4.2

[a] Species identity of plant host in each sampled population.
[b] Number of plant individuals (pooled) processed per population.

Table 2. Comparison of dominant OTUs obtained by high-thoughput sequencing (this study) and cultivation (Glynou *et al.*, 2016) from the same root samples.

Fungus	Sequencing			Cultivation		
	OTU[a]	Proportion (%)[b]	Rank[c]	OTU[d]	Proportion (%)	Rank
Fusarium sp. (Hypocreales)	OTU1	14.2	1	OTU001	8.6	1
Pyrenochaeta sp. (Pleosporales)	OTU2	6.8	2	OTU004	4.1	4
Fusarium sp. (Hypocreales)	OTU3	1.8	13	OTU003	5.4	3
Alternaria sp. (Pleosporales)	OTU4	6.2	3	OTU008	1.6	8
Tetracladium sp. (Helotiales)	OTU5	6	4	OTU012	1.2	12
Alternaria sp. (Pleosporales)	OTU6	5.9	5	OTU002	6.8	2
Cladosporium sp. (Capnodiales)	OTU7	4.1	6	OTU015	0.6	16
Ilyonectria sp. (Hypocreales)	OTU8	0.2	71	OTU005	3.9	5
Cadophora sp. (Helotiales)	OTU9	0.2	72	OTU006	2.7	6
Rutstroemiaceae sp. (Helotiales)	OTU10	2.6	8	OTU040	0.2	40
Pseudorobillarda sp. (Pezizomycotina *Incertae sedis*)	OTU11	1.8	14	n.d.	n.d.	-
Phomopsis sp. (Diaporthales)	OTU12	2.7	7	OTU016	0.7	15
Darksidea sp. (Pleosporales)	OTU13	2.3	9	OTU018	0.5	20
Mycosphaerella sp. (Capnodiales)	OTU14	1.4	16	OTU015	0.6	16
Stemphylium sp. (Pleosporales)	OTU15	1.8	12	OTU026	0.3	27
Ceratobasidiaceae sp. (Cantharellales)	OTU17	1.9	11	OTU270	0	247
Paraphoma sp. (Pleosporales)	OTU19	1.1	19	OTU007	2	7
Alternaria sp. (Pleosporales)	OTU20	1.5	15	OTU011	1.6	9
Sordariomycetes sp. (unclassified Sordariomycetes)	OTU22	1.3	17	OTU080	0.1	92
Neopeckia sp. (Pleosporales)	OTU23	2.3	10	OTU024	0.6	17
Mycena sp. (Agaricales)	OTU24	1.1	18	n.d.	n.d.	-
Monographella sp. (Xylariales)	OTU33	1	20	OTU014	1.1	13

[a] OTUs defined in this study.
[b] Proportion of abundance of each OTU respect to the total abundance.
[c] Rank order of each OTU within the dataset.
[d] OTUs defined by Glynou and colleagues (2016) from fungal isolates.
n.d., not detected.

Figure legends

Figure 1. Comparison of environmental high-throughput sequencing (HTS) data obtained in this study, and cultivation-based data from the same root samples obtained by Glynou and colleagues (2016). a, Relationship between the number of reads in the HTS dataset and the root colonization percentage based on cultivation data. b, Relationship between total richness obtained by HTS (corrected for the unequal read numbers) and cultivation data. Lines in a and b denote the linear regression model of interaction between both variables. c, Overlap in the OTUs detected by HTS and cultivation methods, for the entire study (left) and for individual plant populations (right).

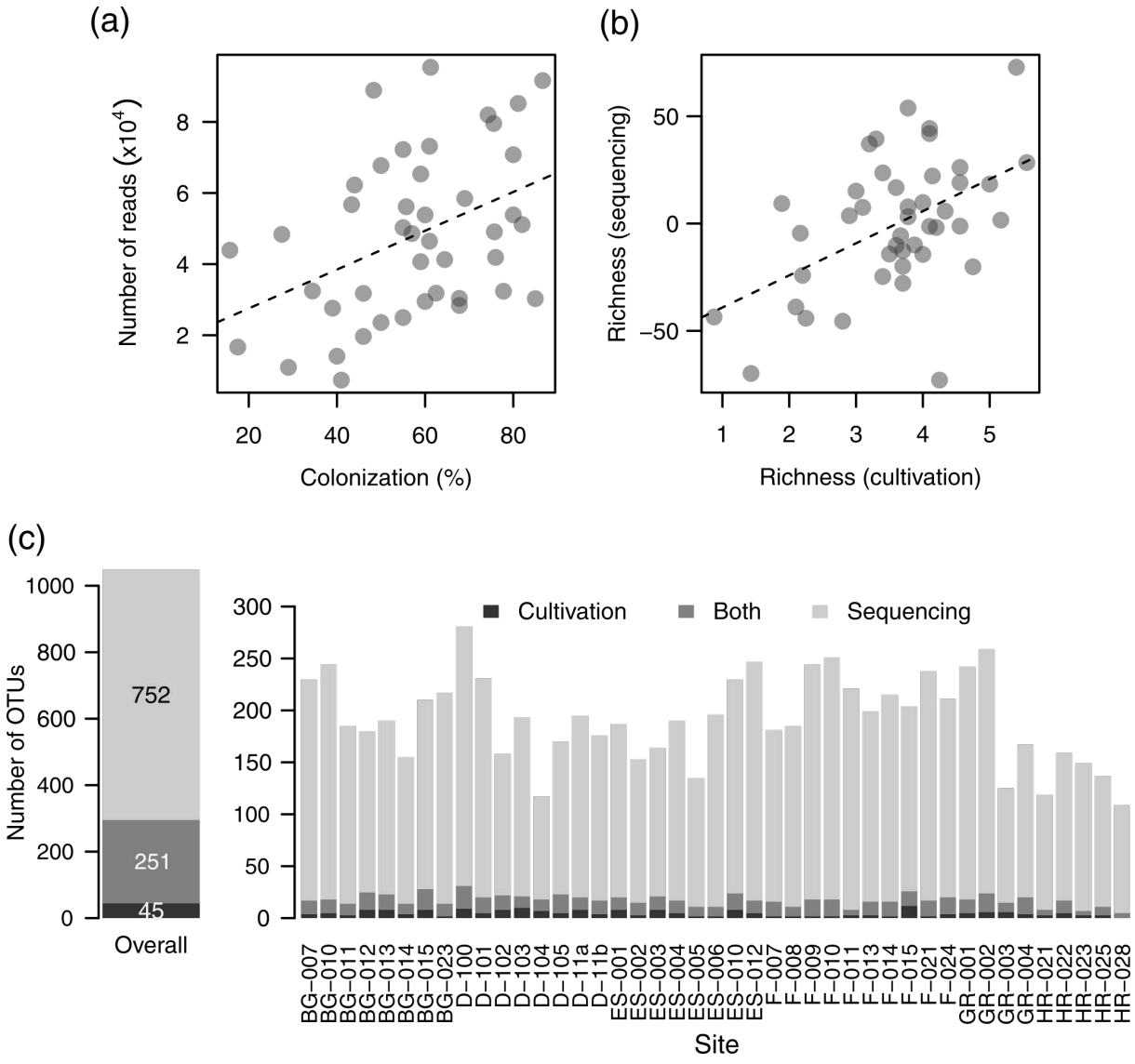


Figure 2. Taxonomic summary of the root-endophytic diversity detected by environmental high-throughput sequencing (HTS) in this study, and by cultivation approaches from the same rootsamples in Glynou and colleagues (2016). Pie-charts show the proportion of OTUs belonging to all fungal phyla detected by HTS (left) and cultivation (right). The bar plot shows the proportion of all fungal orders uncovered by each method. 'Others' in the bar plot comprise both unidentified and *incertae sedis* categories.

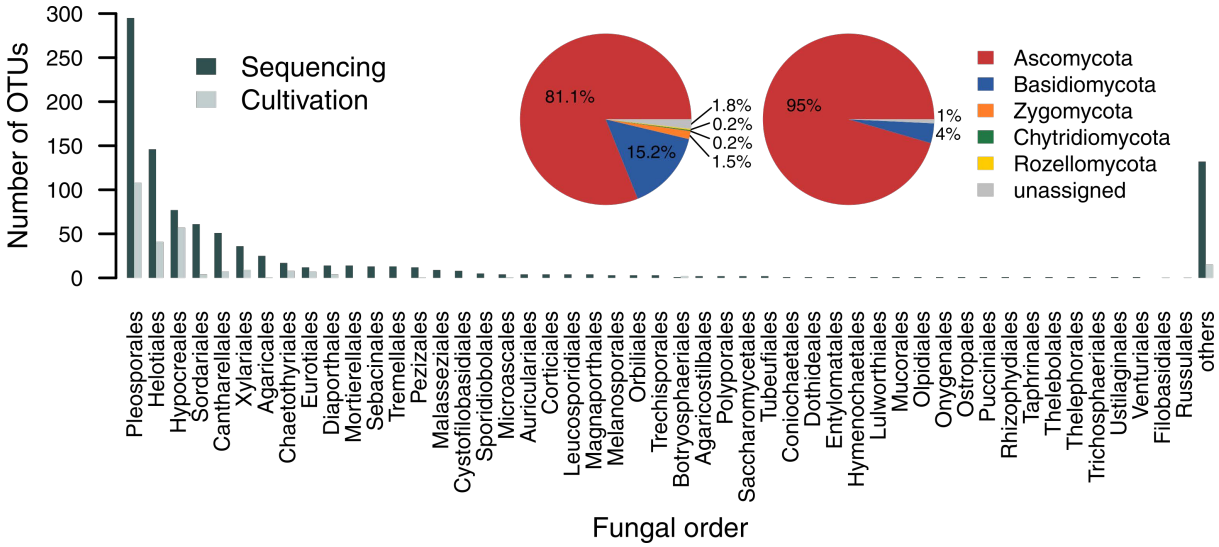


Figure 3. Relationship between richness values in each *Microthlaspi* spp. population with latitude (a) and annual mean temperature (b) at each site. Richness values are shown as partial residuals after correction for unequal read numbers per sample. Lines denote the linear regression model of interaction between both variables.

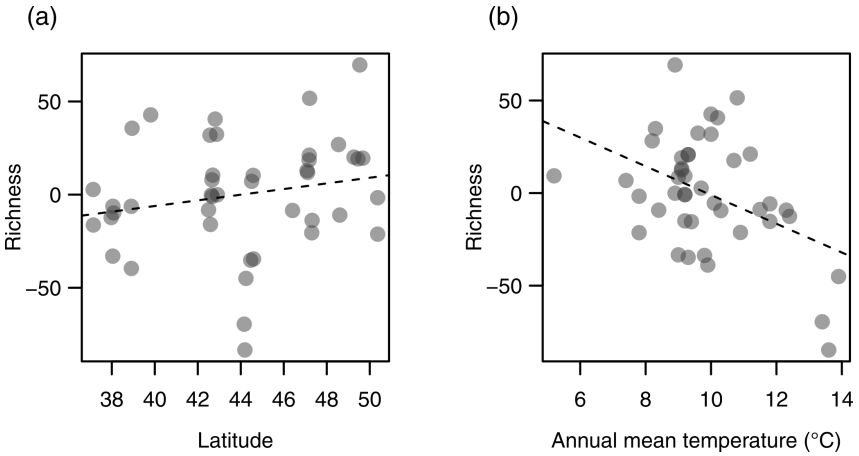


Figure 4. Effect of environmental and spatial factors on community assembly. a, Unconstrained non-metric multidimensional scaling (NMDS) displaying distances among populations based on endophytic community similarity. Arrows show correlations of selected ecological variables (retained after a forward selection) with NMDS axes, in which length and orientation represent the magnitude and direction of the correlation, respectively. Contour lines indicate the association of community structure with latitude. Different point types correspond to identity of the host plant population, with black and white circles denoting *Microthlaspi perfoliatum* and *M. erraticum* populations, respectively, rhombuses mixed populations, and crosses unknown species. b, Euler diagrams displaying the effect of spatial, bioclimatic and soil variables on the community assembly based on variation partitioning. Values indicate the proportion of variation explained by each factor or by combinations of factors. Significant values (P 0.05) are shown in bold face. c, Mantel correlogram showing correlations of community similarity across geographic distance classes in Km, both for the full and the core microbiome datasets. Solid symbols indicate significant (P 0.05) correlations for each distance class. d, Histogram showing the distribution of OTUs range sizes. e, Distribution of range sizes for non-cultivable (NC) and cultivable (C) OTUs. The Box-whisker plot shows the summary statistics for each category (median, quartiles and ranges), and points represent individual OTU values. Abbreviations in a: Bio9, mean temperature of driest quarter; Bio15, precipitation seasonality (CV); pH, soil pH; Cond, soil conductivity; AvK, soil available potassium; PCNM1, PCNM3, PCNM10, spatial factors.

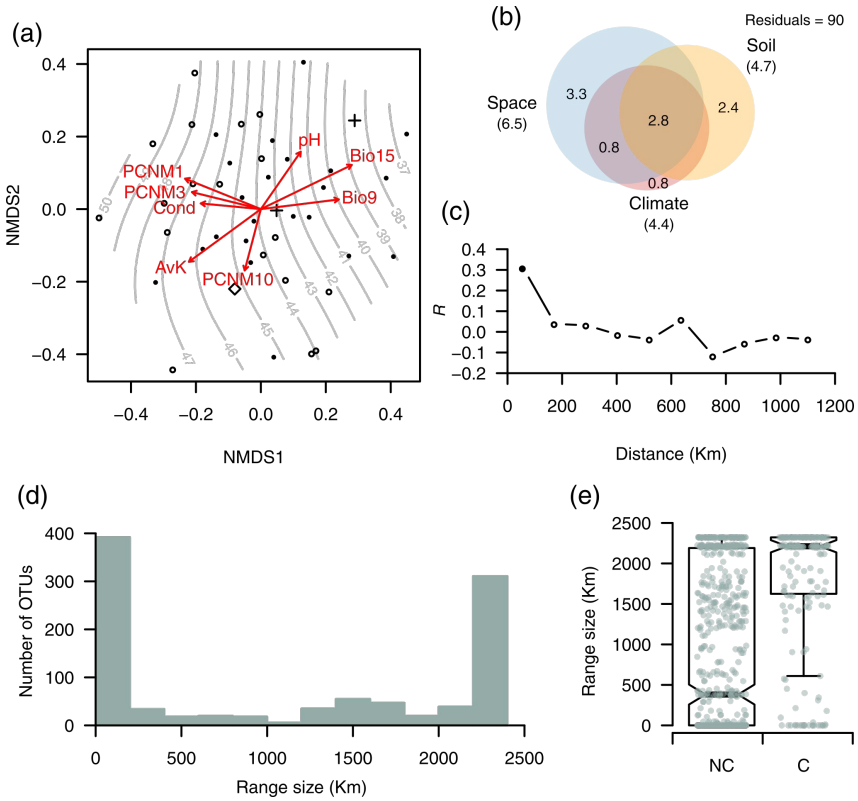
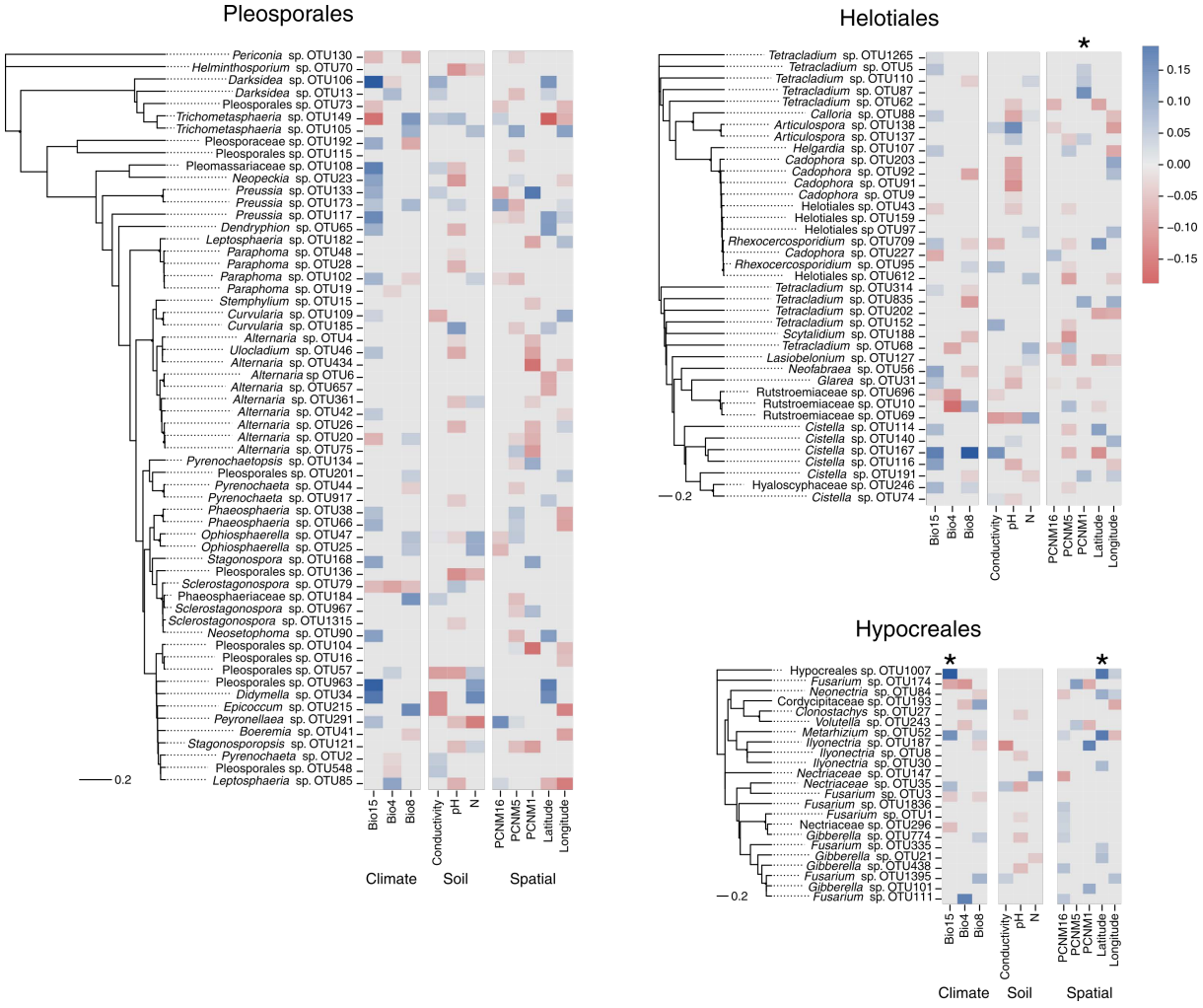


Figure 5. Relationship between individual abundances of core mycobiome OTUs within the most frequent orders in this study (Pleosporales, Helotiales and Hypocreales), and selected climatic, soil and spatial variables. Colors in relationships between OTUs and variables represent coefficients calculated via multispecies generalized linear models (see color key for sign and magnitude), indicating either positive or negative correlations with each factor. Dendrograms displaying the phylogenetic relationships among OTUs are based on maximum likelihood analyses. Asterisks indicate significant and strong (K>0.8, P 0.05) phylogenetic signal in the occurrence of OTUs across specific ecological variables.



Selected Supplementary material

Fig. S1 Quality assessment of Illumina MiSeq sequencing reads for the R1, R2 and the paired sequence dataset.

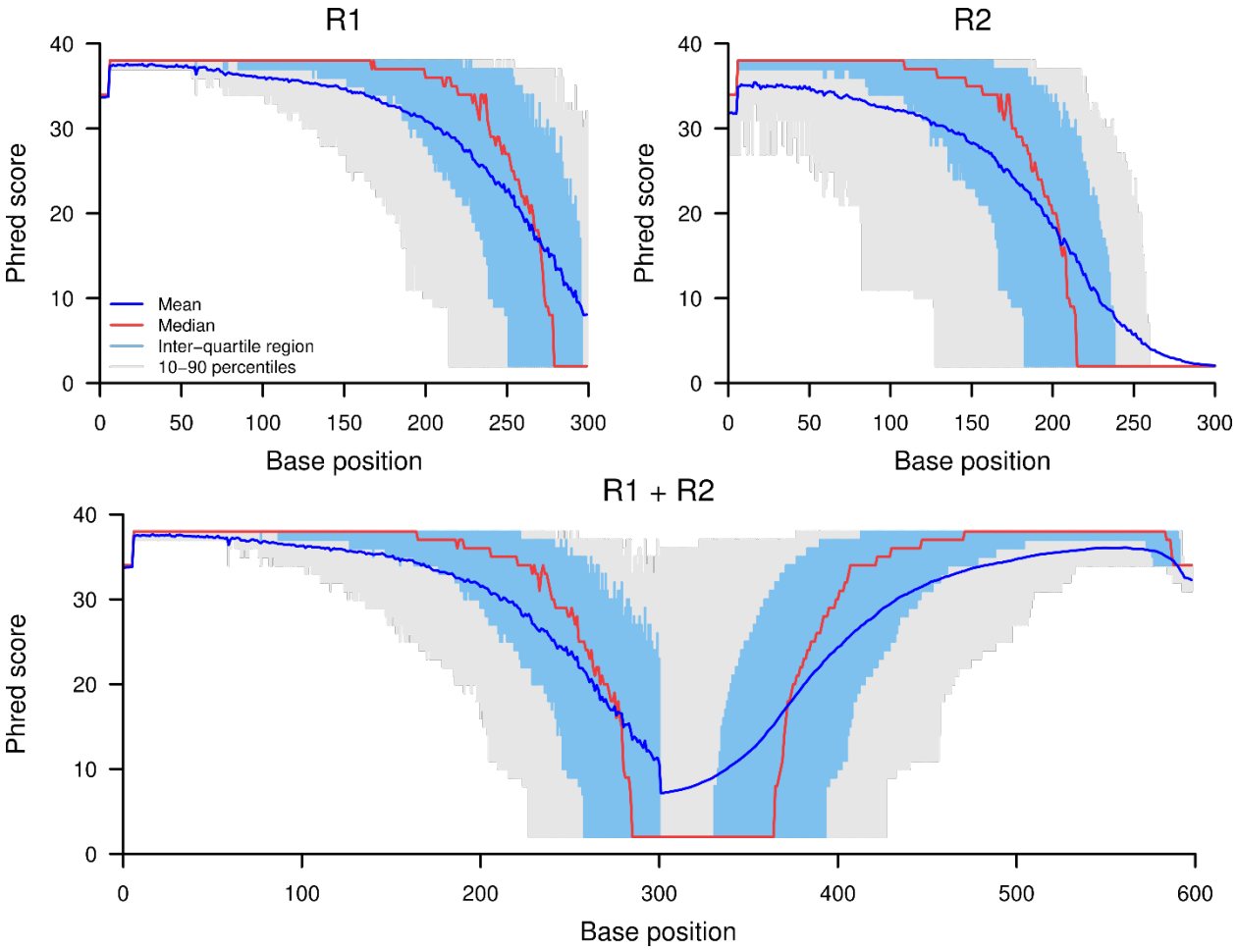


Fig. S2 Percentage of similarities upon BLAST comparisons of OTU rDNA internal transcribed spacer (ITS) sequences with three reference ITS sequence datasets, corresponding to endophytic isolates obtained by Glynou and colleagues (2016), to reference sequences from the UNITE database, or from fungal records deposited in NCBI GenBank.

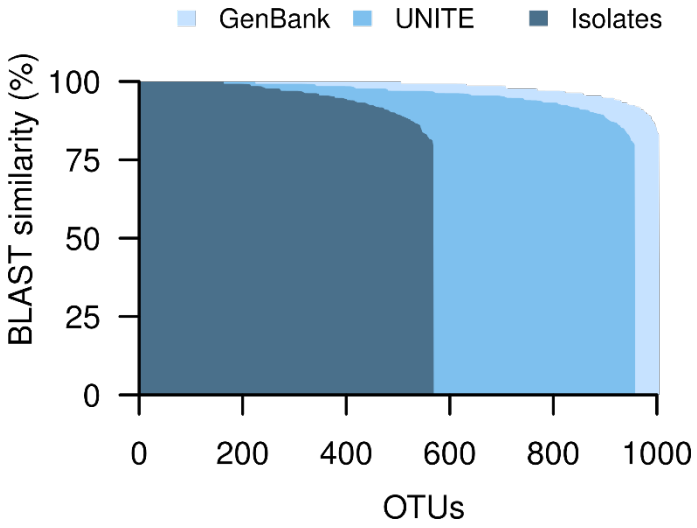


Fig. S3 Summary of Illumina MiSeq sequencing read data obtained in this study. **a**, Histogram showing the distribution of reads per sample (plant population). **b**, Rank-abundance plot displaying the number of reads per OTU.

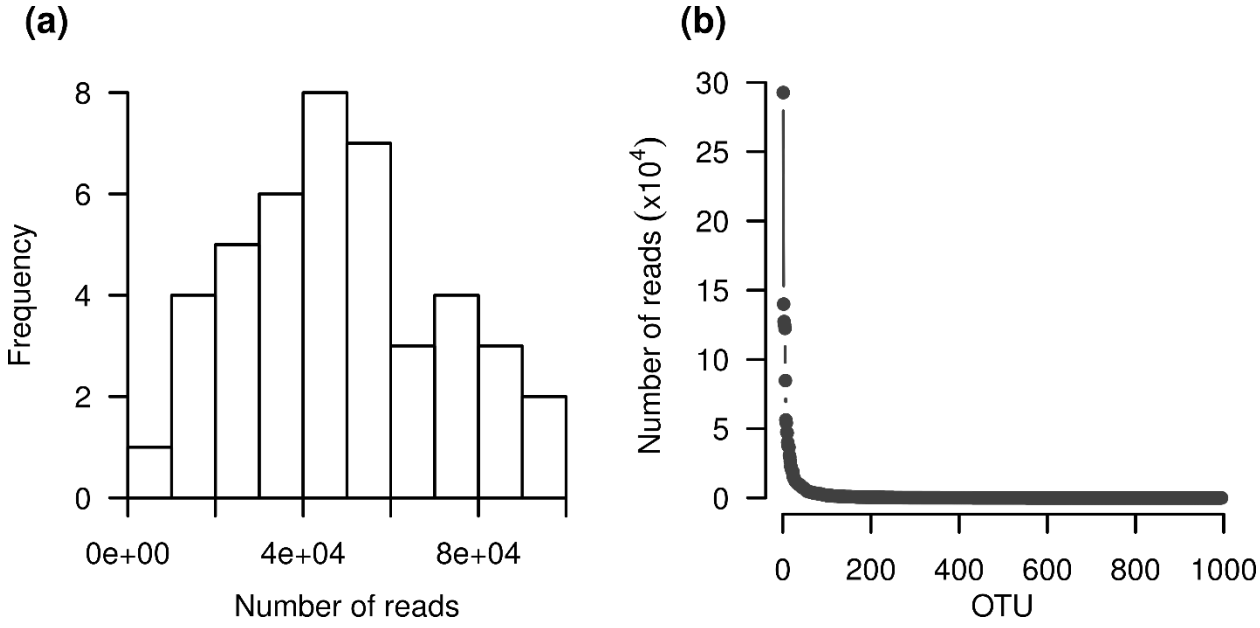


Fig. S4 Detection of OTUs by high-throughput sequencing of a fungal mock-community, containing equimolar amounts of genomic DNA from different fungi isolated by Glynou and colleagues (2016) from the same root samples used in this study.

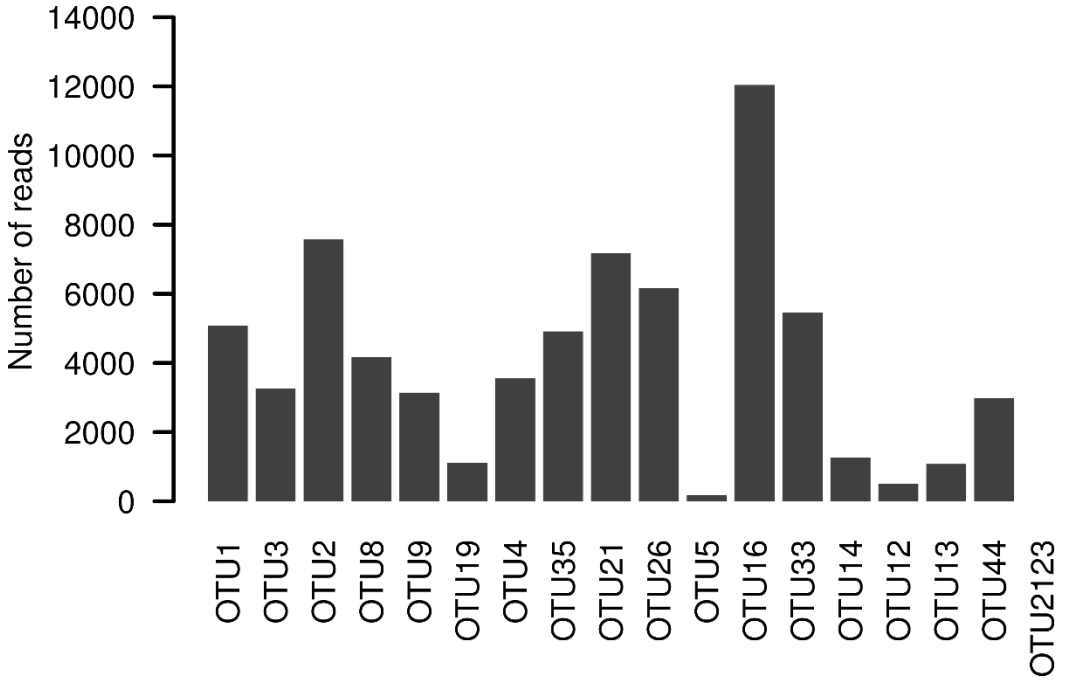


Fig. S5 Detection by high-throughput sequencing of individual OTUs in separate samples containing genomic DNA from dominant fungi isolated by Glynou and colleagues (2016) from the same root samples used in this study. Each line corresponds to reads detected per sample after the clustering of OTUs at 97% ITS sequence similarity using the dataset Glynou and colleagues 2016.

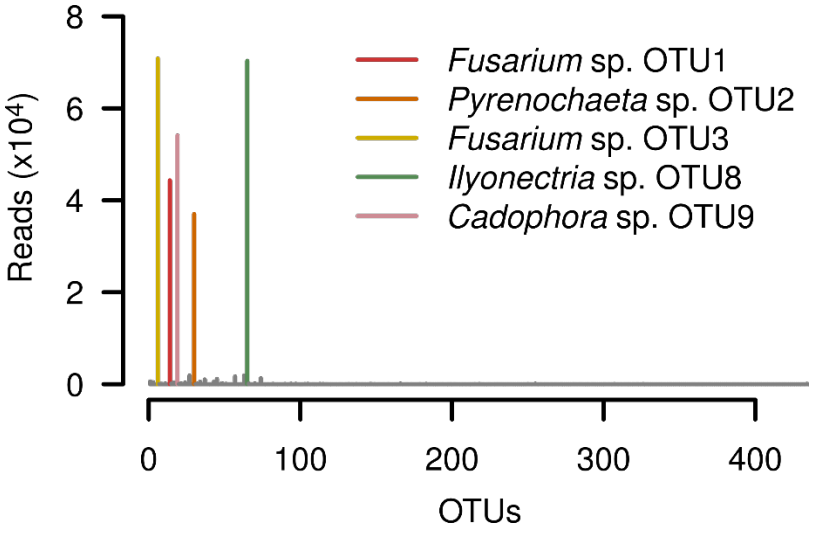


Fig. S6 Rarefaction curves of OTUs accumulation with sequence reads. Curves are calculated for the full and pruned (with single OTU counts per sample removed) datasets in the entire study (a), and for each of the 43 *Microthlaspi* spp. populations individually (b and c for the full and pruned datasets, respectively).

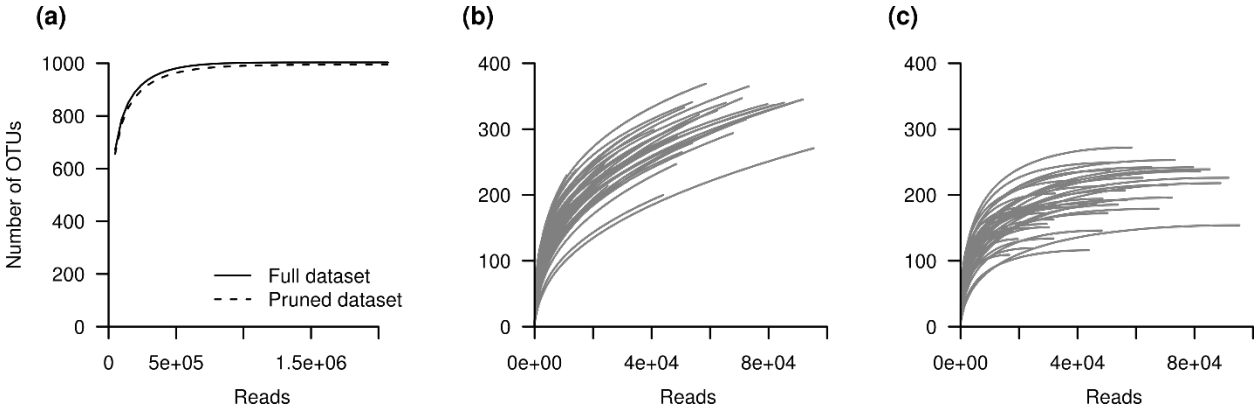


Fig. S7 Relationships between the percentages of cultivable OTUs detected per sample (i.e., number of OTUs detected by both high-throughput sequencing and cultivation methods per sample) and environmental factors. Lines denote the linear regression model of interaction between both variables. Only significant (P<0.05) regressions are shown.

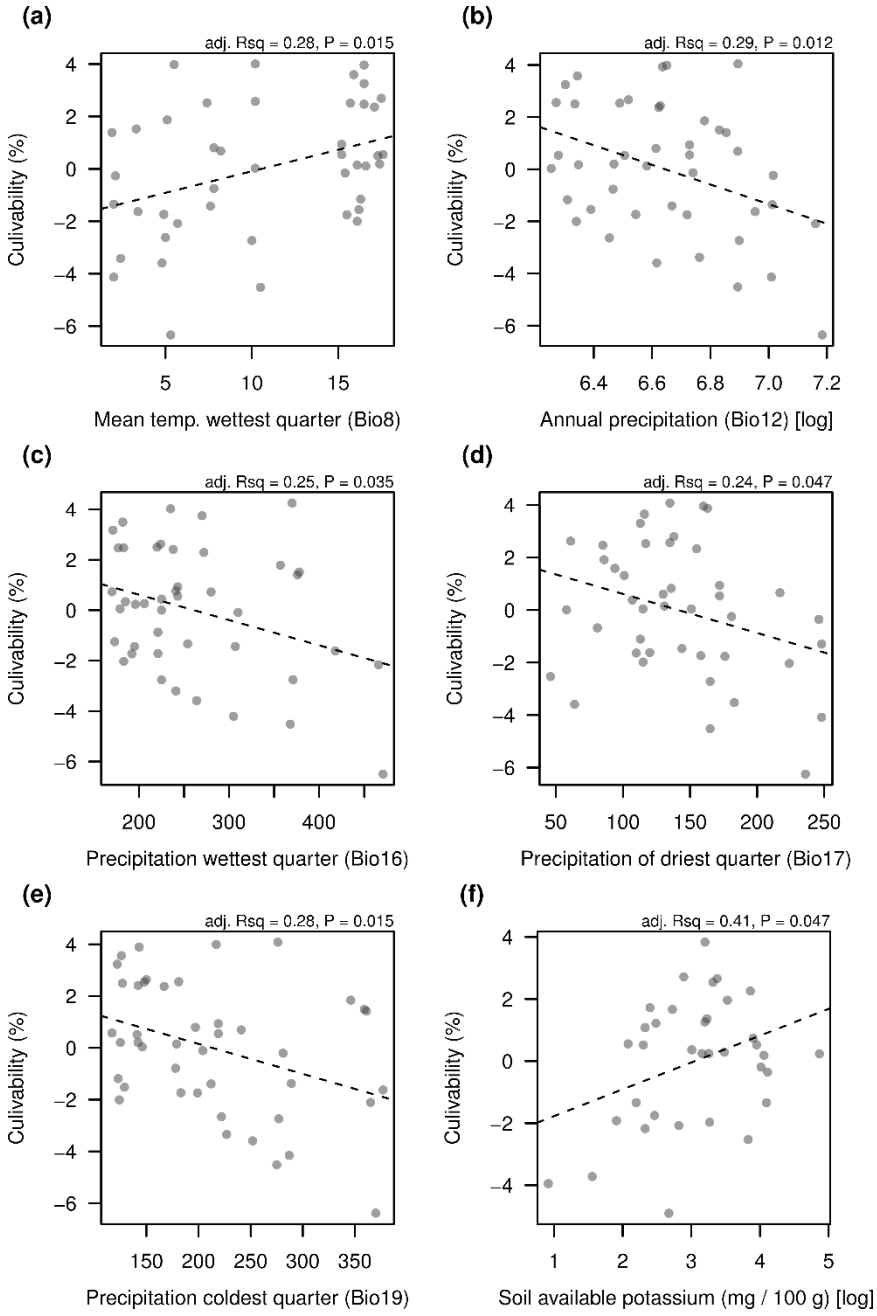


Fig. S8 Relationship between richness and environmental variables. Lines denote the linear regression model of interaction between both variables after correcting for the unequal numbers of sequencing reads per plant populations. Only significant regressions (P<0.05) are shown.

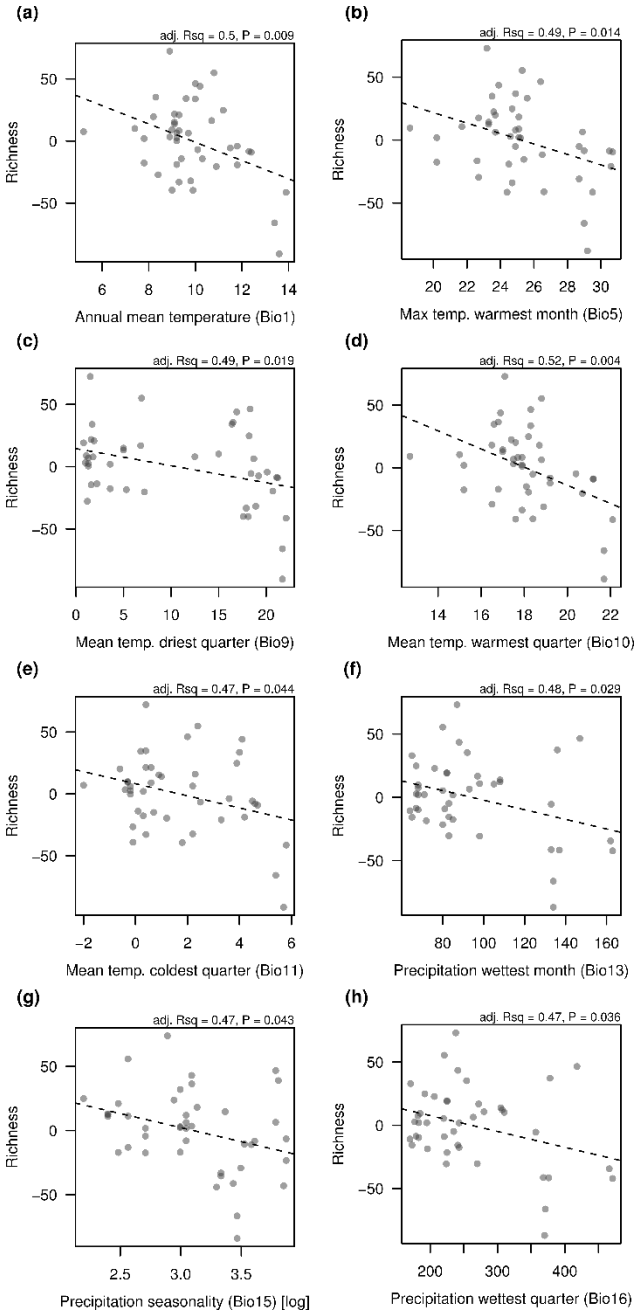
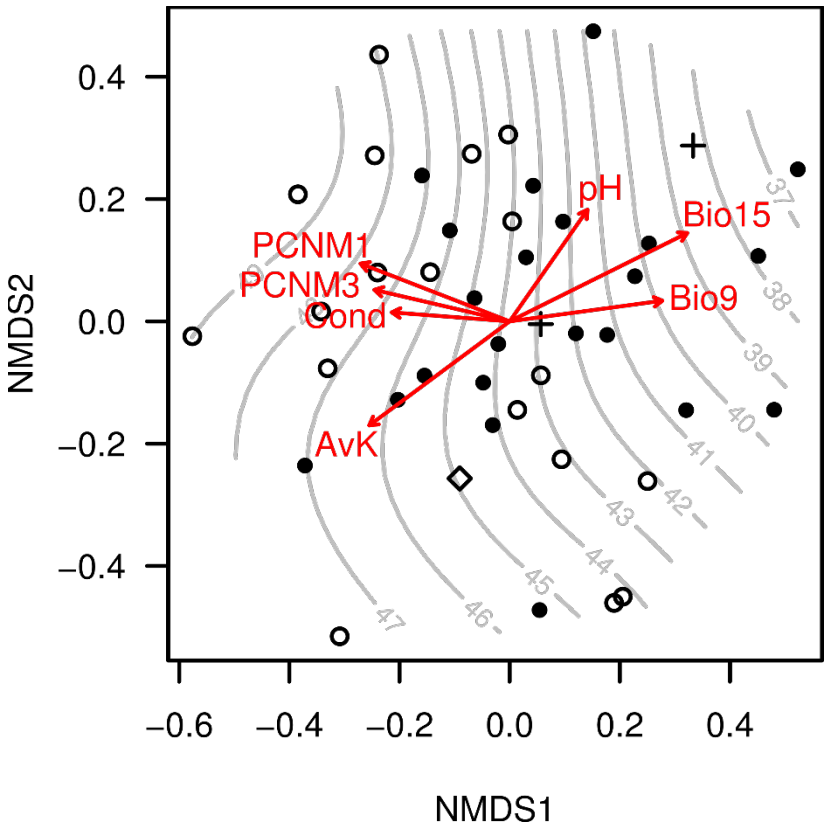


Fig. S9 Unconstrained non-metric multidimensional scaling (NMDS) displaying distances among populations based on community similarity calculated with the Jaccard's incidence-based index. Arrows show correlations of selected ecological variables with NMDS axes (as in Fig. 4a), in which length and orientation represent the magnitude and direction of the correlation, respectively. Contour lines indicate the association of community structure with latitude. Different point types correspond to identity of the host plant population, with black and white circles denoting *Microthlaspi perfoliatum* and *M. erraticum* populations, respectively, rhombuses mixed populations, and crosses unknown species. Abbreviations: Bio9, mean temperature of driest quarter; Bio15, precipitation seasonality (CV); pH, soil pH; Cond, soil conductivity; AvK, soil available potassium; PCNM1, PCNM3, PCNM10, spatial factors.



Chapter 3: Glynou K, Ali T, Kia SH, Thines M, Maciá-Vicente JG. Genotypic diversity in root-endophytic fungi reflects efficient dispersal and environmental adaptation. *Mol Ecol* n/a-n/a.

Anlage 2

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovierende KG: 60%
Co-Author JGMV: 30%
Co-Author MT: 10%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende KG: 80% DNA extraction, amplification (PCR), sequencing, AFLP profiling
Co-Author TA: 5% AFLP profiling
Co-Author JGMV, SHK: 15% DNA extraction, amplification (PCR), sequencing, AFLP profiling

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende KG: 80% collection of sequencing data, colelction of AFLP data, design of figures, submission of sequences to Genbank
Co-Author JGMV: 20% design of figures, submission of sequences to Genbank

(4) zur Analyse und Interpretation der Daten

Promovierende KG: 70% AFLP data analysis, phylogenetic analysis, statistical analysis
Co-Author JGMV: 30% phylogenetic analysis, statistical analysis

(5) zum Verfassen des Manuskripts

Promovierende KG: 70%
Co-Author JGMV: 20%
Co-Authors SHK, TA, MT: together 10%

Zustimmende Bestätigungen der oben genannten Angaben:

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ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Genotypic diversity in root-endophytic fungi reflects efficient dispersal and environmental adaptation

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Abstract

Studying community structure and dynamics of plant-associated fungi is the basis for unravelling their interactions with hosts and ecosystem functions. A recent sampling revealed that only a few fungal groups, as defined by internal transcribed spacer region (ITS) sequence similarity, dominate culturable root endophytic communities of nonmycorrhizal *Microthlaspi* spp. plants across Europe. Strains of these fungi display a broad phenotypic and functional diversity, which suggests a genetic variability masked by ITS clustering into operational taxonomic units (OTUs). The aims of this study were to identify how genetic similarity patterns of these fungi change across environments and to evaluate their ability to disperse and adapt to ecological conditions. A first ITS-based haplotype analysis of ten widespread OTUs mostly showed a low to moderate genotypic differentiation, with the exception of a group identified as *Cadophora* sp. that was highly diverse. A multilocus phylogeny based on additional genetic loci (partial translation elongation factor 1α, beta-tubulin and actin) and amplified fragment length polymorphism profiling of 185 strains representative of the five dominant OTUs revealed a weak association of genetic differences with geography and environmental conditions, including bioclimatic and soil factors. Our findings suggest that dominant culturable root endophytic fungi have efficient dispersal capabilities, and that their distribution is little affected by environmental filtering. Other processes, such as inter- and intraspecific biotic interactions, may be more important for the local assembly of their communities.

KEYWORDS
dispersal limitation, environmental filtering, genotyping, microbial biogeography, root endophytes

1 | INTRODUCTION

Plant-associated microbes have key roles in terrestrial ecosystems as important determinants of plant diversity and community composition (Van der Heijden, Bardgett, & van Straalen, 2008). Fungi are important components of the plant microbiome, but their implication in particular processes is often unclear due to knowledge gaps on their identity, ecology and physiology (Parrent et al., 2010). Understanding the distribution patterns and ecological preferences of plant-associated fungi is necessary to infer their potential ecological roles (Mandyam & Jumpponen, 2005). The large-scale distribution of fungi has been frequently studied (Arnold & Lutzoni, 2007; Cox, Newsham, Bol, Dungait, & Robinson, 2016; Glynou et al., 2016; Higgins, Arnold, Miadlikowska, Sarvate, & Lutzoni, 2007; Tedersoo et al., 2014; Timling, Walker, Nusbaum, Lennon, & Taylor, 2014) and explained within the scope of dispersal limitation and environmental adaptation. However, similar to other microbial groups, results are multi-facetted supporting either environmental filtering (Cox et al.,

2016; Glynou et al., 2016; Queloz, Sieber, Holdenrieder, McDonald, & Grünig, 2011) or dispersal limitation (Salgado-Salazar, Rossman, & Chaverri, 2013) as the main drivers determining fungal distribution. An accurate description of species identity is important, and studies on microbial richness based on operational taxonomic unit (OTU) clustering can be biased due to inaccurate species identification and delimitation (Hibbett et al., 2017). When molecular markers with high genetic resolution are used, they are suitable for detecting intraspecific genetic variability and revealing cryptic species (Bickford et al., 2007), which could otherwise remain undetected with lower resolution markers (Gazis, Rehner, & Chaverri, 2011) or be masked by OTU clustering.

In fungi, intraspecific variability and cryptic speciation has been broadly studied, as evolution within certain lineages can have significant ecological implications. For example, the frequent crop pathogen *Fusarium oxysporum* comprises a large complex of cryptic species and *formae specialis* with differential host specificities (Michielse & Rep, 2009). In arbuscular mycorrhizal (AM) fungi, there is also evidence for specific associations between particular AM fungal genotypes and hosts that influence differences in plant growth (Croll et al., 2008; Wyss, Masclaux, Rosikiewicz, Pagni, & Sanders, 2016). Several studies in fungi show that intraspecific variation is associated with distinct biogeographic patterns (Geml, Tulloss, Laursen, Sazanova, & Taylor, 2008; Taylor, Turner, Townsend, Dettman, & Jacobson, 2006). The intraspecific variability among individuals can also be associated with differences in functional traits and therefore is considered to be related to environmental adaptations (Johnson, Martin, Cairney, & Anderson, 2012) and to affect ecosystem processes (Hughes, Inouye, Johnson, Underwood, & Vellend, 2008).

Here, we study the genetic differences within groups of fungi widespread in root endophytic communities, originating from a recent study on the continental-scale distribution of fungal endophytes from the brassicaceous genus *Microthlaspi* (Glynou et al., 2016). The communities were dominated by a few fungal phylotypes (hereafter referred to as OTUs) with a widespread presence across the sampled area, but which had distinct biogeographic patterns associated with specific environmental conditions. Isolates within these OTUs displayed variations in their morphological and physiological characters and in their interaction with plants (Kia et al., 2017). In addition, strains of a same OTU isolated from different geographic locations have been shown to express differential profiles of production of secondary metabolites (Cheikh-Ali et al., 2015). Altogether, these results suggest that phenotypic variation among strains may be potentially linked to genotypic differences within OTUs, which could even represent different species (Gazis et al., 2011). Moreover, potential genetic differences may be related to specific environments or follow spatial patterns associated with geographic or environmental distance. A correlation of genetic similarity with geographic proximity would imply dispersal limitations driving variation (Peay, Kennedy, & Talbot, 2016). Conversely, a correlation with similarity in environmental conditions would suggest that environmental filtering plays a key role in the accumulation of genetic variation (Cox et al., 2016).

In Glynou et al. (2016), we relied on sequencing of the rDNA internal transcribed spacer region (ITS) for OTU delimitation and classification. This marker is widely used in studies of fungal diversity (Arnold & Lutzoni, 2007; Maciá-Vicente, Ferraro, Burruano, & Lopez-Llorca, 2012; Taylor et al., 2014) as ITS is an optimal universal barcoding region because it is easy to amplify, offers efficient identification rates in multitaxon ecological studies (Schoch et al., 2012), and it is well represented in sequence databases, thus offering useful comparative information. However, reliance only on ITS sequence similarity thresholds to delimit OTUs can mask genetic differences and introduce biases in species delimitation (O'Donnell et al., 2015; Peay et al., 2016; Schoch et al., 2012).

In this study, we aimed to assess the genotypic variation within OTUs of widespread root endophytic fungi defined by Glynou et al. (2016), some of which had been already found by an independent study on the same host plant (Keim, Mishra, Sharma, Ploch, & Thines, 2014). We also aimed to identify potential associations of the genotypic variation with geographic distance and environmental conditions, which could indicate processes of dispersal limitation and environmental filtering. A detailed analysis of the patterns of intragroup genetic diversity in root endophytes will help to draw a link between their distribution and their ecological functions.

2 | MATERIALS AND METHODS

2.1 | Fungal material

Fungal material originated from a previous study (Glynou et al., 2016) on the diversity of endophytic fungi associated with roots of the brassicaceous plants *Microthlaspi erraticum* and *Microthlaspi perfoliatum* (Ali, Runge et al., 2016; Ali, Schmuker et al., 2016). In that study, a collection of 2,006 fungal cultures was generated after the cultivation of surface-sterilized root segments from plants in 52 populations, distributed across northern Mediterranean and central Europe (Figure 1). Endophytes were maintained as pure cultures in the Integrative Fungal Research (IPF) culture collection hosted at Goethe University (Frankfurt am Main, Germany). The ITS region of all isolates was sequenced and used for molecular characterization following procedures in Glynou et al. (2016). In brief, genomic DNA was extracted from cultures with the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) using a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA). The ITS region was amplified and sequenced with the primer pair ITS1F/ITS4 (Gardes & Bruns, 1993; White, Bruns, Lee, & Taylor, 1990) and used for taxonomic classification with the Naïve Bayesian classifier (Wang, Garrity, Tiedje, & Cole, 2007) as implemented in MOTHUR v1.34.4 (Schloss et al., 2009), based on comparisons with the UNITE database of reference fungal ITS sequences (Kõljalg et al., 2013). Isolates were clustered into OTUs according to 97% ITS pairwise sequence similarities using the BLASTCLUST algorithm (Altschul, Madder, & Schäfer, 1997). To get an insight into the ITS sequence variability of the dominant fungal groups, we selected the sequences from all isolates belonging to the ten most abundant OTUs (based on their isolation

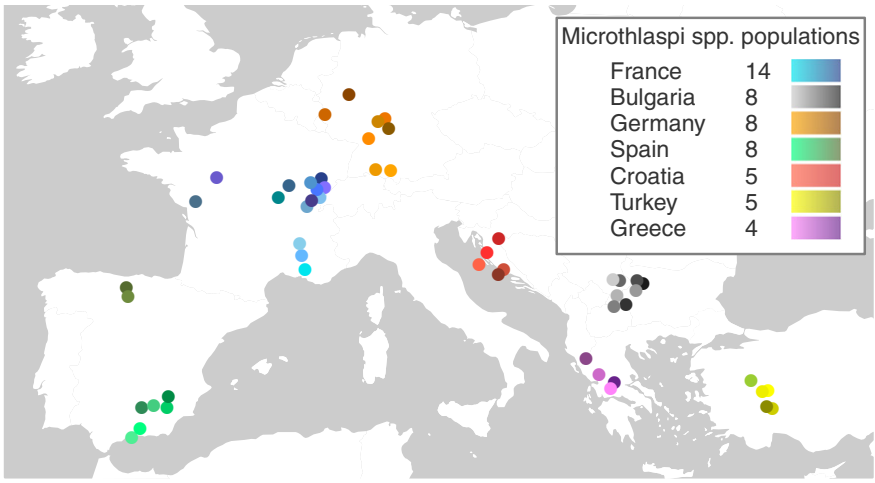

FIGURE 1 Locations of origin of the root endophytic fungi in this study. Points indicate the approximate locations of each *Microthlaspi* spp. population sampled by Glynou et al. (2016)

frequency) and used them to construct haplotype networks (Table 1). Additionally, representative isolates from the five most abundant OTUs were used for multilocus sequencing and amplified fragment length polymorphism (AFLP) profiling (Table 1). For the selection of these isolates, we aimed at covering as many sampling sites as possible, and each site with more than one isolate. A subset of isolates were grown on potato dextrose agar (PDA; Applichem, Darmstadt, Germany) and photographed to illustrate macromorphological variation in colony aspect.

We retrieved data on the geographic location and the bioclimatic and soil conditions from where each isolate was obtained, as provided by Glynou et al. (2016). Bioclimatic variables were therein obtained from the WorldClim (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) and the Consortium for Spatial Information (CGIAR-CSI; Trabucco & Zomer, 2009) data sets. Likewise, soil physicochemical variables were obtained by Glynou et al. (2016) by direct measurements on soil samples collected alongside plant material. The details of all isolates and environmental variables used in this study are provided in Table S1.

2.2 | Haplotype analysis

We performed a haplotype network analysis using the ITS sequences of the ten most abundant OTUs obtained by Glynou et al. (2016), to assess differences among sequences not captured by OTU classifications at >97% sequence similarity (Table 1). Haplotypes representing unique ITS sequences were used to construct networks summarizing the relationships and connections between haplotypes, using function "haploNet" in the package PEGAS (Paradis, 2010) of R v3.3.1 (R Core Team 2016). Networks are based on Hamming distances (Hamming, 1950) among sequences with pairwise deletion of missing data, and return the most probable links among haplotypes, the

TABLE 1 Details of the fungal endophytes included in this study, showing the number of samples used for haplotype network analyses, multilocus phylogenies and amplified fragment length polymorphism (AFLP) profiles

			Haplotype networks[b]		Multilocus phylogeny[c]				
OTU[a]	Proposed identification	Order	Isolates	Haplotypes	ITS	*TEF-1α*	*TUB*	*ACT*	AFLP[d]
OTU001	*Fusarium tricinctum* species complex	Hypocreales	249	20	49	49	39	48	48
OTU002	*Alternaria tellustris*	Pleosporales	247	32	51	51	47	51	51
OTU003	*Fusarium oxysporum* species complex	Hypocreales	144	16	34	33	30	27	19
OTU004	*Pyrenochaeta* sp.	Pleosporales	120	10	27	27	27	25	25
OTU005	*Dactylonectria* aff. *macrodidyma*	Hypocreales	110	10	24	24	24	23	24
OTU006	*Cadophora* sp.	Helotiales	94	54	—	—	—	—	—
OTU007	*Paraphoma* sp.	Pleosporales	85	32	—	—	—	—	—
OTU008	*Alternaria* aff. *alternata*	Pleosporales	56	6	—	—	—	—	—
OTU009	*Fusarium redolens* species complex	Hypocreales	47	7	—	—	—	—	—
OTU010	*Fusarium incarnatum-equiseti* species complex	Hypocreales	46	5	—	—	—	—	—

[a]Classification into operational taxonomic units as defined by >97% ITS sequence similarity, as described by Glynou et al. (2016).
[b]Number of sequences included in haplotype network analyses, and of haplotypes generated for each OTU.
[c]Number of sequences from each OTU included in phylogenetic analyses, from either the rDNA internal transcribed spacers (ITS), or the genes for the translation elongation factor 1α (*TEF-1α*), beta-tubulin (*TUB*) and actin (*ACT*).
[d]Number of isolates from each OTU processed for AFLP profiles.

number of mutational steps for each link and the probability of each link calculated based on clade parsimony following Templeton, Crandall, and Sing (1992). The function also returns a list of alternative links among haplotypes, but they were not considered here for simplicity.

We used a combination of rarefaction and extrapolation of haplotype accumulation curves per OTU to estimate the coverage of our isolates' sampling, using the R package INEXT (Hsieh, Ma, & Chao, 2016). The method is based on Hill numbers (effective numbers of species) to estimate completeness in species sampling, in which the parameter q indicates species richness ($q = 0$), "typical" species ($q = 1$) and dominant species ($q = 2$; Chao et al., 2014), but can also be applied to estimate genotypic diversity.

2.3 | DNA amplification and sequencing

A total of 185 DNA extracts from a selection of isolates belonging to the five dominant OTUs found by Glynou et al. (2016) were used for sequencing of the partial translation elongation factor 1α (*TEF-1α*), beta-tubulin (*TUB*) and actin (*ACT*) gene regions (Table 1). These loci were selected based on their frequent use for taxonomic purposes in fungi, as well as on their demonstrated value as universal barcoding regions (Couch & Kohn, 2002; Lawrence, Gannibal, Peever, & Pryor, 2013; O'Donnell et al., 2015; Stielow et al., 2015; Woudenberg, Groenewald, Binder, & Crous, 2013). Amplification reactions were carried out using a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany). Reactions were performed in a final volume of 21 μl, containing 1 μl of DNA template, 1× PCR Buffer (VWR International, Darmstadt, Germany), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, 0.8 mM bovine serum albumin (BSA) and 0.5 U Taq polymerase (VWR International). *TEF-1α* was amplified using primers EF1-983F and EF1-2218R (Rehner & Buckley, 2005) with the following amplification conditions: an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 45 s, 52°C for 40 s and 72°C for 60 s, and a final extension step of 72°C for 5 min. For amplification of *ACT*, we used primers ACT-512F and ACT2Rd (Carbone & Kohn, 1999; Groenewald et al., 2013) and ACT1Fd and ACT1Rd (Groenewald et al., 2013), with the following conditions: 94°C for 4 min, 30 cycles of 94°C for 45 s, 52°C for 40 s and 72°C for 90 s, and a final step of 72°C for 5 min. Amplification of *TUB* from strains assigned to *Fusarium tricinctum* species complex (*F. tricinctum* onwards) and *Dactylonectria* aff. *macrodidyma* was performed using primers Bt2a and Bt2b (Glass & Donaldson, 1995), and with primers Btubk1F (GTCACTACACTGAGGGTGCC) and Btubk1R (CTTGGGGTCGAACATCTGCT) for the other fungi. PCR conditions for amplification of *TUB* consisted of an initial step of 94°C for 4 min, 35 cycles of 94°C for 45 s, 52°C for 40 s and 72°C for 45 s, and a final step of 72°C for 5 min. PCR products were sequenced in both directions by the sequencing laboratory of the Biodiversity and Climate Research Centre (Frankfurt am Main, Germany). Sequences were assembled using the "merger" tool of EMBOSS v6.6.0.0 (Rice, Longden, & Bleasby, 2000). All newly generated sequences have been deposited in GenBank (see Table S1 for Accession nos).

2.4 | AFLP profiling

AFLP fingerprinting was used to complement multilocus sequencing analyses. The method has proven useful for detection of genetic variation within fungal species (Al-Hatmi et al., 2016; Majer, Mithen, Lewis, Vos, & Oliver, 1996). We obtained AFLP profiles from 167 isolates representing the five selected OTUs (Table 1) following Vos et al. (1995) with minor modifications. Approximately 225 ng of genomic DNA from each isolate was digested at 37°C for 150 min in 20-μl reactions containing 1.25 U Mse1 (NEB, Frankfurt am Main, Germany) and 0.63 U EcoR1 (Thermo Fisher Scientific). Restricted fragments were then ligated in 5-μl reactions containing 5 μM of Mse1 adapters and 0.5 μM of Ecor1 adapters and used for different amplifications. A first nonselective amplification was performed in 21-μl reactions with 1× PCR Buffer, 2 mM $MgCl_2$, 1 μM Ea primer (GACTGCGTACCAATTCA), 2 μM Mc primer (GATGAGTCCTGAGTAAC), 0.2 mM dNTPs, 0.9 U Taq DNA polymerase and 3 μl of ligated samples. Temperature cycles included an initial step of 94°C for 3 min, 26 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 105 s and a final step of 72°C for 5 min. The resulting reactions were diluted sevenfold and used for selective amplification in three different PCR runs with forward labelled primers E1 (FAM-GACTGCGTACCAATTCAGC; 1 μM), E2 (VIC-GACTGCGTACCAATTCAGG; 1 μM), E9 (PET-GACTGCGTACCAATTCAAC; 1 μM) and reverse nonlabelled primer M9 (GATGAGTCCTGAGTAACGA; 1 μM). Selective PCRs were performed in 12 μl, with the same reagents as in the nonselective PCRs but including 0.3 mM BSA. PCR cycles consisted of an initial denaturation of 94°C for 3 min followed by 26 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by one cycle of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. Duplicate AFLP profiles from a random selection of 10% of the strains were obtained to account for reproducibility of the profiling process (Bonin, Ehrich, & Manel, 2007). Samples were analysed by the sequencing laboratory of the Biodiversity and Climate Research Centre. The resulting AFLP profiles were analysed for each OTU separately. Electropherograms were analysed for peak detection using PEAKSCANNER v1.0 (Applied Biosystems Inc., Waltham, MA, USA). The assignment of bins was performed using the R package RAWGENO (Arrigo, Holderegger, & Alvarez, 2012) with a minimum scoring at 50 bp. Finally, matrices of presence/absence of bins for all isolates were obtained and used for further statistical analyses. The matrices with AFLP data are provided in Table S2.

2.5 | Phylogenetic analyses

Sequences of the ITS, *TEF-1α*, *ACT* and *TUB* were independently processed for each OTU. Sequences in each data set were aligned with the G-INS-I algorithm of MAFFT v7.123b (Katoh & Standley, 2013), trimmed with GBLOCKS v0.91b (Castresana, 2000) and then used to construct Maximum Likelihood trees using RAXML v8.2.0 (Stamatakis, 2014) with 1,000 bootstrap replicates, using the GTRGAMMA model of nucleotide substitution and rate heterogeneity. As outgroups, sequences from this study were selected, belonging to the closest

phylogenetically related OTU that was outside of the one being tested. The proportion of phylogenetically informative characters for each locus was obtained from the respective alignments using SEAVIEW v4.4.2 (Gouy, Guindon, & Gascuel, 2010). Congruence between trees built with individual loci for each OTU was tested with function "CADM.global" of the R package APE (Paradis, Claude, & Strimmer, 2004). The congruence test and the tree topologies for each locus showed no support of conflict. Thus, we concatenated the sequence alignments for each OTU using FASCONCAT v1.0 (Kück & Meusemann, 2010) and generated ML concatenated trees, performed as for individual loci but allowing independent estimates for each sequence partition. Phylogenetic trees were edited and visualized using APE. All alignments and trees have been deposited in TreeBASE under Accession no. 20544.

2.6 | Statistical analyses

We used the plant populations from which the isolates were isolated as a proxy to represent differences in geographic locations and environmental conditions across sampling sites. Chi-squared tests were used to investigate the distribution of particular frequent haplotypes across sites, as compared to the overall distribution of their respective OTUs. Distance matrices based on the Jaccard's index were calculated from AFLP binary profiles using the "vegdist" function of the R package VEGAN (Oksanen et al., 2015). We used principal coordinates analysis (PCoA) to visualize distances of AFLP profiles among isolates within each OTU. Distance matrices were generated from the multilocus phylogenetic trees using the function "cophenetic" of APE. Euclidean geographic distances in km among sampling sites were obtained with function "spDists" of the SP package (Pebesma & Bivand, 2012). Euclidean distances were also obtained for the bioclimatic and soil variable data sets using scaled values with R function "dist." We investigated the relationships between the isolates' genetic differences and factors related to geographic and environmental distances using Mantel tests (Legendre & Legendre, 2012) for independent data sets, using functions "mantel" and "mantel.correlog" of VEGAN. Partial Mantel tests were used to subtract the effect of either geographic, bioclimatic or soil distances from correlations, using *vegan*'s function "mantel.partial."

3 | RESULTS

Haplotype networks, including a total of 1,197 ITS sequences, were built to represent the intragroup variability within the ten most represented OTUs obtained by Glynou et al. (2016) (Figure 2). Several OTUs were rather homogeneous, displaying one central haplotype with a very high frequency, and a small number of singleton or low-frequency haplotypes. Others, including *Fusarium tricinctum* (OTU001), *Cadophora* sp. (OTU006), *Paraphoma* sp. (OTU007) and *Fusarium incarnatum-equiseti* species complex (OTU010), displayed a different pattern. For *F. tricinctum*, *Paraphoma* sp. and *F. incarnatum-equiseti*, most sequences clustered within two and three main haplotypes with high frequency, respectively. On the other hand, *Cadophora* sp. displayed a singular pattern of haplotypes structured in a complex network, in which almost 50% of the sequences represent unique haplotypes (Figure 2). Estimates of haplotypes' sampling coverage using accumulation curves agreed with the patterns in haplotype networks, with values of or above 90% haplotypes' coverage in all cases except for OTU006 (Fig. S1). Accumulation curves of ITS variants showed a sufficient representation of frequent (q = 1 and 2), but not of rare haplotypes (q = 0; Fig. S1). There was no apparent geographic clustering of haplotypes, because the main clusters were represented by isolates from a variety of countries and locations.

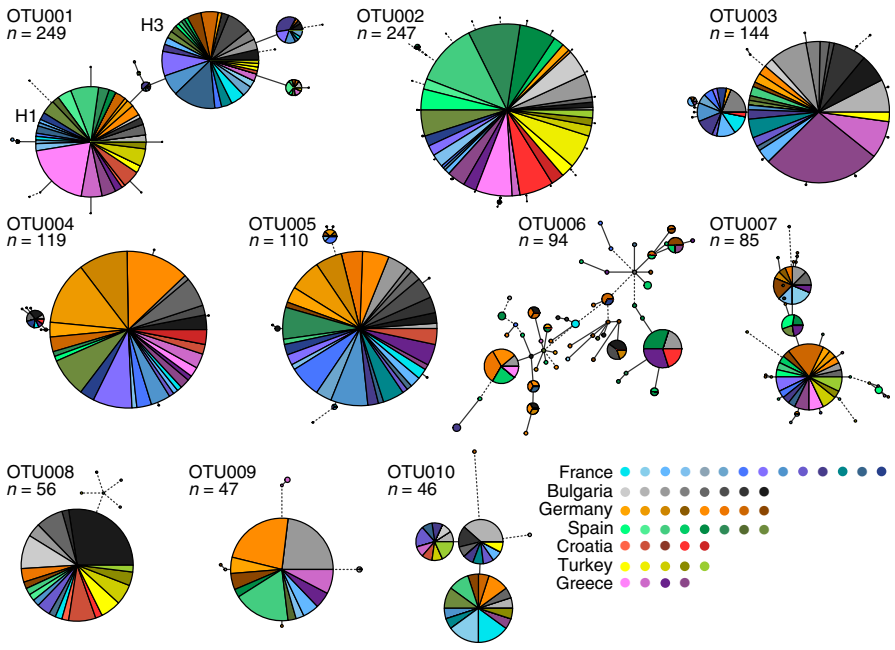

FIGURE 2 Haplotype networks representing ITS sequence variability within the operational taxonomic units (OTUs) most frequently found by Glynou et al. (2016) (see Table 1). Each pie represents a haplotype, with size relative to the total number of sequences included. Sectors in pies indicate the proportion of each haplotype at different geographic locations (see colour key). Shades of the same colour represent different populations within the same country. Dashed lines in links between pies indicate a probability smaller than .99 based on clade parsimony. The two most frequent haplotypes in OTU001 (*Fusarium tricinctum*), H1 and H3, are indicated

Only the two major haplotypes of *F. tricinctum* appeared to be somewhat associated with latitude according to chi-squared tests, which supported a preferential occurrence of haplotypes H1 and H3 in southern and northern sites, respectively ($p \leq .001$; Fig. S2).

We selected 185 isolates from the five most abundant OTUs to assess possible genotypic variation via multilocus phylogeny and AFLP profiling (Table 1). Strains in some of these OTUs displayed an evident macromorphological variation (Figure 3). For example, isolates of *F. tricinctum* were variable in colony colour, structure of the mycelium and colony size. In contrast, cultures of *Alternaria tellustris* were homogeneous in their production of dark mycelium and fast growth (Figure 3). Overall, 710 sequences were used for multilocus phylogenetic analyses after sequencing of three loci in addition to ITS (Table 1; Figure 4). The proportion of phylogenetically informative characters was low for all loci in all OTUs, with a median value of 0.6% of all characters in each alignment, and never exceeding 10% (Table S3). Phylogenetic analyses were performed using concatenated sequence partitions within each OTU, after assessing congruent topologies in individual trees. The defined clades in all trees mostly had low bootstrap support values (<90%), with a few exceptions (Figure 4). *Fusarium tricinctum* displayed the largest variability, with several well supported clusters comprising multiple isolates, which followed an analogous pattern to that found for haplotypes H1 and H3 in the haplotype network (Figure 4). *Fusarium oxysporum* species complex (*F. oxysporum* onwards) and *Pyrenochaeta* sp. showed two clades each with bootstrap values above 90%, but they were only represented by two strains. *Alternaria tellustris* and *Dactylonectria* aff. *macrodidyma* displayed the most homogeneous trees. Separation of isolates in these cases did not reflect separation based on the geographic location.

Similar patterns to those found with the phylogenetic trees were observed in PCoAs representing the variation in AFLP profiles (Figure 4). AFLP analyses for OTU001–OTU005 yielded 77, 113, 31, 50 and 105 bins, respectively. The distribution of bin frequencies was similar for *F. tricinctum*, *A. tellustris* and *D.* aff. *macrodidyma*, but not for *F. oxysporum* and *Pyrenochaeta* sp., possibly due to the small number of bins generated (Fig. S3). PCoA analyses explained 27%–40% of the total variation for each OTU. In *F. tricinctum*, we found again a significant separation between isolates from haplotypes H1 and H3 (Figure 4).

Overall, partial Mantel correlations between genetic divergence and geographic distances among isolation sites were low for both sequencing and AFLP data (Tables 2 and S4). *Alternaria tellustris* was the only OTU for which genetic differences calculated from phylogenetic and AFLP data agreed in a significant correlation with geographic distance (Table 2). Environmental distance as represented by bioclimatic factors (Table S1) had a significant positive correlation only in the multilocus data sets for *F. tricinctum* and *Pyrenochaeta*

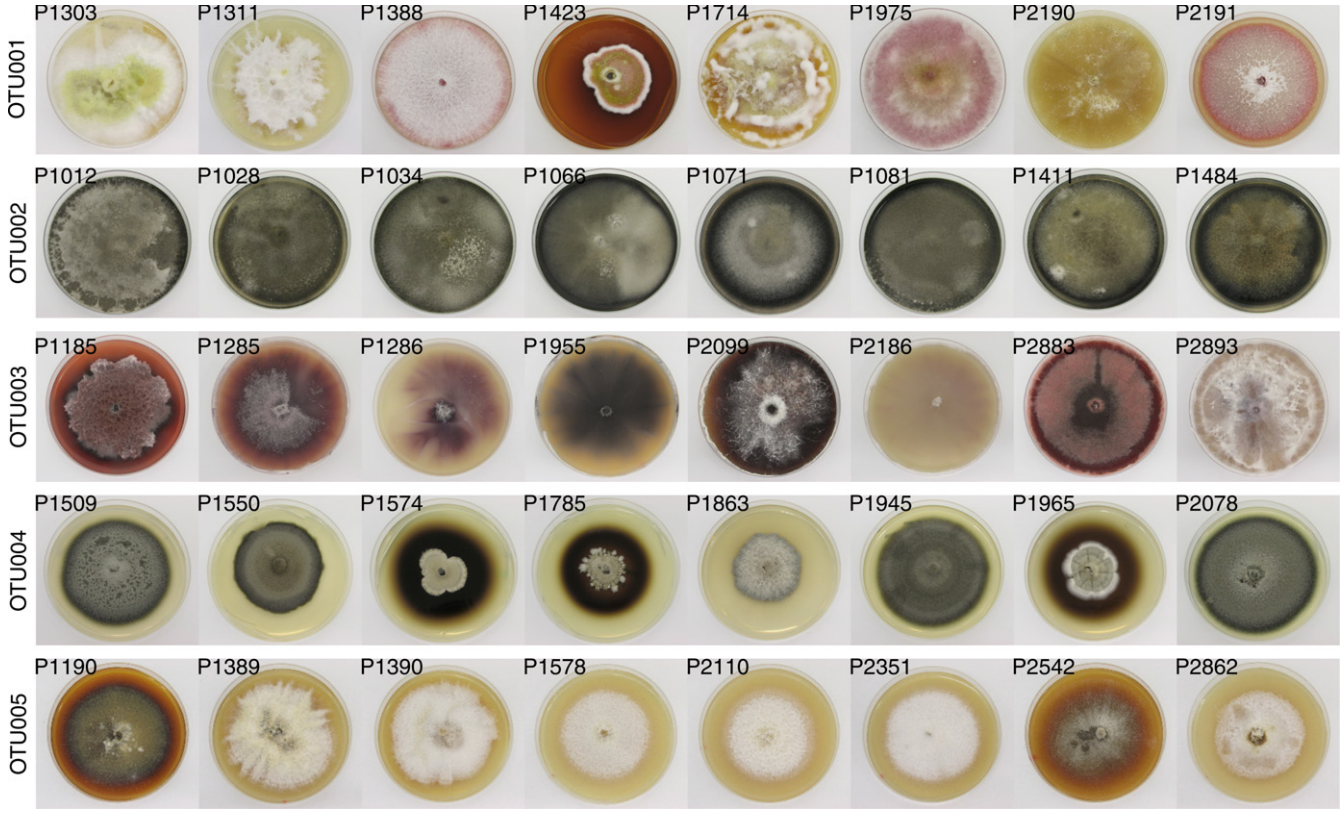

FIGURE 3 Macromorphological variation of representative isolates of the five operational taxonomic units (OTUs) selected for this study. Photographs show pure cultures of a selection of isolates for each OTU after one month of growth on potato dextrose agar. See Table S1 for details on each isolate. Numbers at the top left corner of each picture indicate the isolate number

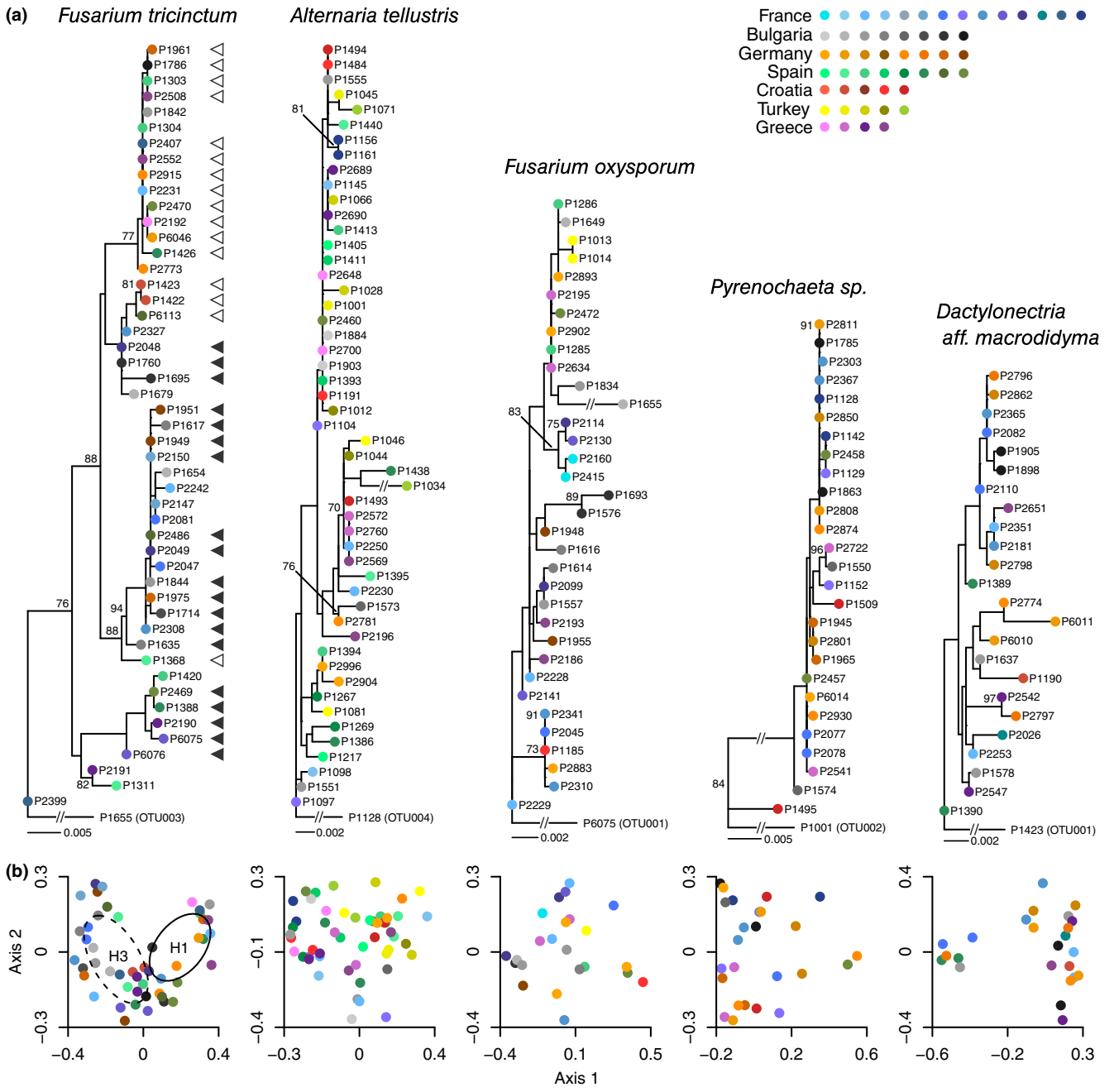

FIGURE 4 Within- operational taxonomic unit (OTU) genetic relationships of isolates represented by multilocus phylogenetic (a) and amplified fragment length polymorphism (AFLP) data (b). Trees in (a) represent topologies based on Maximum Likelihood (ML) analyses of concatenated alignments of ITS, *TEF-1α*, *TUB* and *ACT* nuclear DNA regions. Node labels correspond to bootstrap values after ML analysis with 1,000 bootstrap replicates; only ML values equal or higher than 70% are shown. In the tree for *Fusarium tricinctum*, the two main haplotypes defined on ITS sequences, H1 and H3, are indicated with empty and solid triangles, respectively. Plots in (b) correspond to principal coordinates analyses (PCoA) based on similarities of AFLP profiles. Each point corresponds to one isolate. Ellipses in the PCoA of *F. tricinctum* delimit the 95% confidence intervals for isolates within haplotypes H1 (solid line) and H3 (dashed line). Colour of points in trees and PCoAs represent the locations of origin of isolates (see colour key)

sp., whereas soil factors were not associated with genotypic variation in any fungus (Table 2). Significance and magnitude of partial correlations were maintained in full Mantel tests without controlling for the effect of geographic and environmental distances, and in partial Mantel tests where the effect of bioclimatic factors was corrected with soil factors and vice versa (Table S4). The effect of the geographic and environmental distances across distance classes was investigated with use of Mantel correlograms (Figure 5). *Fusarium tricinctum*, *A. tellustris*, *F. oxysporum* and *D.* aff. *macrodidyma* showed either no or weak spatial patterns across classes both for phylogenetic and AFLP data (Figure 5). Only isolates of *Pyrenochaeta* sp. showed a phylogenetic resemblance that rapidly declined beyond

TABLE 2 Partial Mantel tests of correlations between genetic distance and geographic, bioclimatic and soil distances

	Geography | Climate[a]		Geography | Soil		Climate | Geography		Soil | Geography	
	R^b	p^c	R	p	R	p	R	p
Fusarium tricinctum								
Phylogeny	0.027	.269	**0.098**	**.048**	**0.102**	**.045**	−0.049	.743
AFLP	0.01	.427	0.03	.239	0.04	.244	0.03	.345
Alternaria tellustris								
Phylogeny	**0.109**	**.019**	0.01	.401	−0.099	.924	−0.218	.985
AFLP	**0.127**	**.004**	−0.01	.544	−0.005	.531	0.06	.222
Fusarium oxysporum								
Phylogeny	0.037	.314	0.068	.239	−0.102	.882	0.03	.399
AFLP	−0.08	.812	−0.09	.724	0.05	.31	−0.1	.716
Pyrenochaeta sp.								
Phylogeny	−0.155	.942	**0.227**	**.042**	**0.289**	**.028**	0.084	.226
AFLP	0.07	.248	0.04	.324	−0.05	.663	−0.05	.614
Dactylonectria aff. *macrodidyma*								
Phylogeny	−0.064	.74	−0.073	.652	−0.065	.638	−0.052	.656
AFLP	**0.14**	**.04**	0.12	.132	−0.07	.724	0.05	.288

AFLP, amplified fragment length polymorphism.
[a]Matrix for correlation with genetic distances | matrix for controlling in partial Mantel test.
[b]Partial Mantel statistic.
[c]*p*-values after Bonferroni corrections for multiple comparisons. Values in bold denote significantly supported correlations ($p \leq .05$).

700 km. This pattern, however, was not supported by AFLP similarity. Mantel correlograms for bioclimatic and soil factors did not show any significant pattern for both phylogenetic and AFLP data (Figure 5).

4 | DISCUSSION

We have used haplotype network analysis, multilocus phylogenetic inference and AFLP profiling to assess the genotypic variability within groups of fungal endophytes dominant in roots of *Microthlaspi* spp., which had a widespread distribution at a continental scale (Glynou et al., 2016). Our results show that the genetic variation across isolates is rather low within these fungal groups, and it does not follow a clear biogeographic pattern associated with either geographic distance or environmental conditions. This is in agreement with previous studies on root-associated fungi (Queloz et al., 2011; Rosendahl, Mcgee, & Morton, 2009), which showed no correlation between genetic diversity within populations and spatial or environmental factors at the continental scale. Our findings suggest that the endophytic groups herein studied have efficient dispersal abilities and/or are not subject to strong adaptive pressure due to adaptations to the abiotic environment.

The three methodologies we used to assess genetic differences among isolates within OTUs agreed in a rather low differentiation within the five most represented OTUs found by Glynou et al. (2016). Indeed, estimates of haplotypes' sampling coverage indicated that analysing a relatively low number of isolates is sufficient to detect the dominant haplotypes within most OTUs across the sampled area. In the case of *Fusarium tricinctum* (OTU001), the ITS-based haplotype analysis generated two frequent haplotypes that were further supported by the multilocus phylogeny and the AFLP analysis. The two haplotypes showed a differential latitudinal distribution, with one being more frequent in southern and the other in northern regions. However, this partitioned occurrence does not necessarily reflect a dispersal limitation or a restricted environmental tolerance, given that both haplotypes were found across the entire range of the OTU. Instead, it is suggestive of vicariant closely related lineages with a mutual exclusion due to competition for the host. The local predominance of one haplotype over the other could result from slight preferences for extant conditions favouring a particular population, thus providing a competitive advantage for the pioneer colonization of roots (Peay et al., 2016). A particular case was that of isolates classified as *Cadophora* sp. (OTU006), which presented a complex haplotype network with a large number of haplotypes with low individual frequencies. *Cadophora* and allied genera are known to be species-rich and to exhibit a high variability in their ITS sequences (Taylor et al., 2014). Therefore, we cannot discard the possibility that the classification of isolates within this OTU based on 97% ITS sequence similarity lumped together several closely related species widespread across geographic locations.

Genetic differences among the isolates of the five dominant endophytic OTUs were not significantly structured spatially, supporting that their distribution is not significantly constrained by geographic distance. Only phylogenetic and AFLP distances in *Alternaria tellustris* showed a consistent association with geography

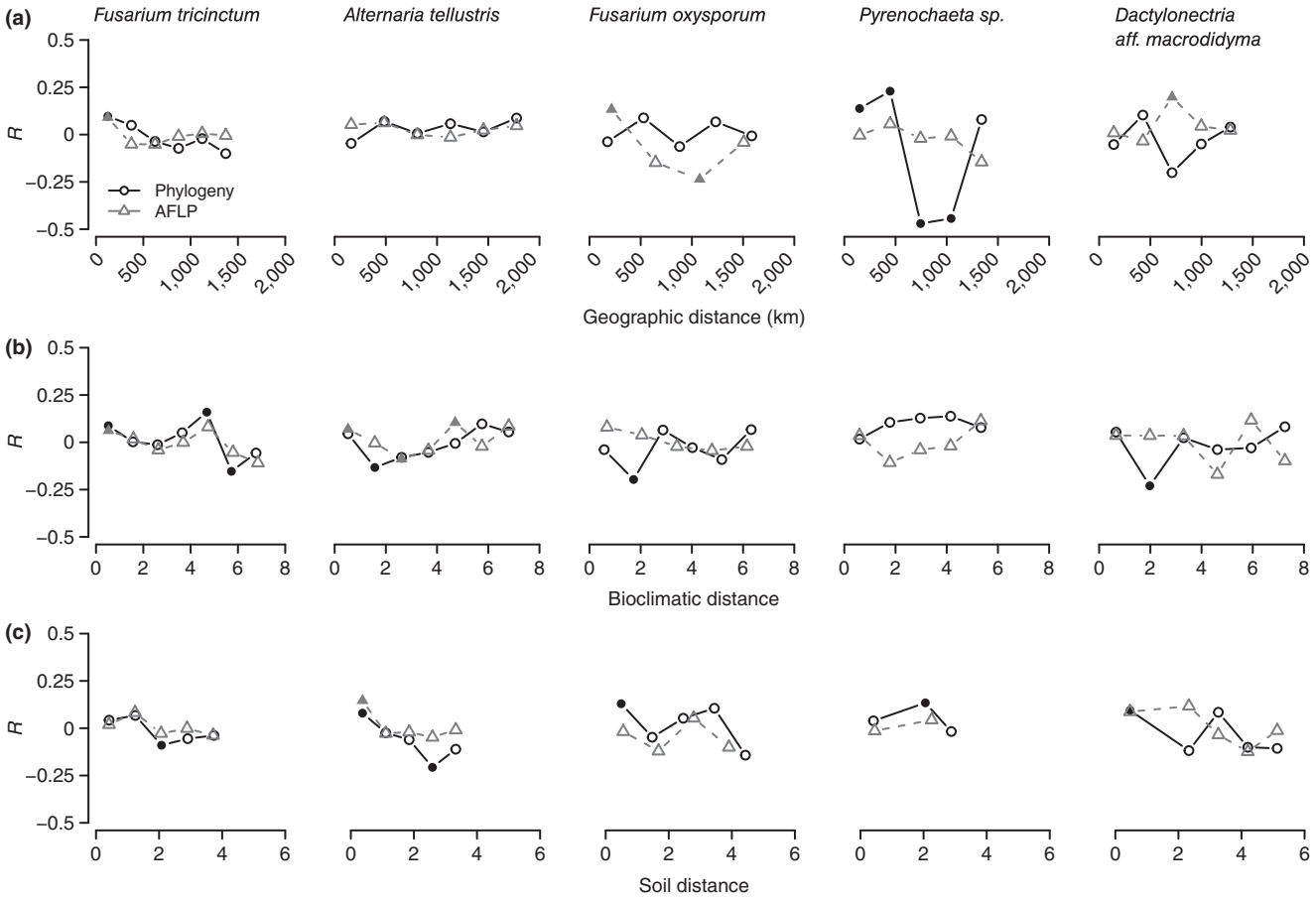

FIGURE 5 Mantel correlograms for the five OTUs selected showing correlations of genetic similarity across geographic distance classes (a) and environmental relative distance classes, as calculated from bioclimatic (b) and soil (c) data. Black and grey lines represent genetic similarities calculated from phylogenetic and amplified fragment length polymorphism (AFLP) data, respectively. Solid symbols denote significant ($p \leq .05$) correlations for each class

in partial Mantel tests, although this was not evident in correlograms across distance classes. The general lack of spatial structure is in agreement with our previous results on the overall presence of OTUs, even though some OTUs with a potentially more limited dispersal capability were also found (Glynou et al., 2016). This provides clues on the dispersal capabilities of these fungal groups, and on their ability to adapt and thrive in new environments. It has been stated that long-distance dispersal of fungal propagules is limited (Brown & Hovmøller, 2002), supported by findings that cosmopolitanism in fungi is not as common as previously thought (Peay et al., 2016). However, similar findings to ours have been previously shown for other root-associated fungi (Davison et al., 2015; Hazard et al., 2013; Queloz et al., 2011; Rosendahl et al., 2009). The ability of fungi to come in contact and mix over large spatial distances is related to their particular biological characteristics. For example, species of *Fusarium* and *Dactylonectria* can produce three kinds of spores and are able to disperse through air and water (Chaverri, Salgado, Hirooka, Rossman, & Samuels, 2011; Nelson, Dignani, & Anaissie, 1994; Palmero et al., 2011). *Alternaria* spp. produce airborne conidia that can disperse over thousands of kilometres by themselves, or carried away by plant material (Hjelmroos, 1993; Mims & Mims, 2004; Skjøth et al., 2016). *Pyrenochaeta* spp., on the other hand, produce microsclerotia in soil and within plant roots and are not frequent in air (Grove, 1987), which would theoretically restrict their distribution. Other factors that facilitate a widespread distribution in these fungi could be linked to human activities (Rosendahl, 2008), as they are closely related to well-known crop pathogens which could be distributed through transfer of seeds or other plant material. The lack of geographic barriers to the movement of these root endophytes might also help to maintain a constant gene flow within their populations, thus hindering genetic divergence through isolation (Rosendahl, 2008).

In most cases, there was no strong evidence of environmental filtering determining the genetic structure of populations. A certain degree of positive correlation with bioclimatic conditions was found for *Pyrenochaeta* sp., which was independent of geographic distance. However, results as inferred from multilocus and AFLP data were inconsistent and no clear significant patterns were evident in the correlograms across distance classes. While this result may suggest a slight effect of the local environment in selecting particular genotypes within this OTU, further assessments with more sensitive markers able to better resolve genomic polymorphisms would be

necessary to conclude on this possibility. Phylogeny and AFLP results for *Pyrenochaeta* sp. often were discrepant, and hence, it is possible that they did not accurately represent genotypic variation. While both methodologies generally agree, lack of congruence can arise from low signal/noise ratios in AFLP data or an insufficient amount of data (Koopman, 2005). The lack of a clear footprint of environmental filtering in the genetic structure of endophytic populations is noteworthy, taking into account that our sampling covered an area with marked bioclimatic differences. Nevertheless, these conditions are adequate for growth of the host *Microthlaspi* spp., which in turn may be the main factor determining the occurrence of these fungi. Their widespread distribution across space and habitat conditions could be partly explained by their high specialization towards colonization of plant roots (Buckling, Wills, & Colegrave, 2003), which could overrule the effect of other environmental factors.

Morphological differences as well as functional variation across strains of the endophytic OTUs studied were observed (Kia et al., 2017), but they were not reflected by their genetic relationships. It is possible that the strain-specific plant interactions frequently observed in endophytes of a same species (e.g., Kia et al., 2017; Tellenbach, Grünig, & Sieber, 2011) are associated with particular changes in specific genes important for the symbiosis, rather than entailing genome-wide variations easily detected with commonly used profiling methods. For example, pathogenicity in *Fusarium* species is often related to the presence of specific, motile chromosomes carrying sets of virulence genes (Ma et al., 2010; Rep & Kistler, 2010), or to individual genes subject to strong adaptive pressure (Sperschneider et al., 2015). Kia et al. (2017) found that, although plant-fungal interactions are influenced by fungal phylogeny, they also have a large variability associated with recent divergences over evolutionary time, which supports a quick adaptation of fungal symbionts to local conditions. Another explanation for the phenotypic differences between fungal cultures may be the symbiotic association with fungal viruses, which have been reported to interact with fungal endophytes (Herrero, Márquez, & Zabalgogeazcoa, 2009). Mycoviruses can cause morphological abnormalities in their hosts' mycelium (Aoki et al., 2009; Ghabrial & Suzuki, 2009; Rohwer & Youle, 2011) and also affect plant–fungal interactions. Their effect is related to induction of hypovirulence in pathogenic fungi (Pearson, Beever, Boine, & Arthur, 2009; Yu et al., 2010), or enhancement of the mutualistic function of endophytes (Márquez, Redman, Rodriguez, & Roossinck, 2007). Phenotypic variation among genetically similar populations, however, could also be explained by accumulated epigenetic changes in functional genes induced by environmental conditions (Martienssen & Colot, 2001; Verhoeven, vonHoldt, & Sork, 2016).

It is conceivable that isolates originating from the same plant population could represent clonal individuals of a same genetic background. This could have led to an inflation of the significance values in correlation tests due to repeated measures (Peres-Neto & Legendre, 2010), even after corrections by geographic distance in partial Mantel tests. However, as we did not uncover strong correlations between genetic distances and geography or environmental conditions, we can be confident that this bias has little influence on our conclusions. Finally, while our selection of methods to assess genotypic variability are common in population studies and considered of sufficient genetic resolution (Taylor & Fisher, 2003), subtler divergences could potentially be detected with use of high-throughput profiling methods (Branco et al., 2015; Ellison et al., 2011; Wyss et al., 2016). Their enhanced resolution could help assess fine-grained genetic variability undetected in our study.

5 | CONCLUSIONS

Our findings support the hypothesis of an efficient dispersal of dominant culturable root endophytic fungi, as well as of a lack of environmental filtering in the genetic structure of their populations. This favours the mixing of distant fungal genets in endophytic assemblages, restricting genetic divergence at a continental scale. The co-occurrence of different genotypes of an endophytic species implies that they can colonize simultaneously the same host tissues, and thus, the final impact on plant fitness likely is the result of the combination of the phenotypic traits they express. It is conceivable that the genetic diversity observed is due to different host preferences apart from *Microthlaspi* species, that is, that while the contribution of the abiotic environment and spatial distance is only minor factor explaining the diversification of the endophytes, their interaction with various host plants and the adaptation to their biotic niche are the main drivers of genetic differentiation. At the local scale, biotic processes affecting interactions among fungal genotypes, such as facilitation or competition, might be the main determinants of the outcome of the interaction with plants.

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DATA ACCESSIBILITY

- DNA sequences are deposited in GenBank with accessions KT268320–KT270306, KX361424–KX361944, KX682392–KX682396. Accession nos are provided with the associated metadata in Table S1.
- DNA alignments and phylogenetic trees are available in TreeBASE under accession 20544.
- Matrices with the binary AFLP data are provided in Table S2.

- These data have been uploaded to Dryad. https://doi.org/10.5061/dryad.bh6k6.

AUTHOR CONTRIBUTIONS

K.G., M.T. and J.G.M.V. designed the research. K.G., A.T. and S.H.K. conducted the research. M.T. and J.G.M.V. contributed reagents and analytical tools. K.G. and J.G.M.V. analysed the data and drafted the manuscript. All authors contributed to the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Selected Supplementary material

Figure S1. Sample-size and coverage based accumulation curves of haplotypes within the ten OTUs included in this study (left and right plot for each OTU, respectively), accounting for all (q = 0), 'typical' (q = 1), and dominant (q = 2) haplotypes. Solid lines indicate rarefaction and dashed line indicate extrapolation of diversity, and shaded areas delimit 95% confidence intervals for each curve.

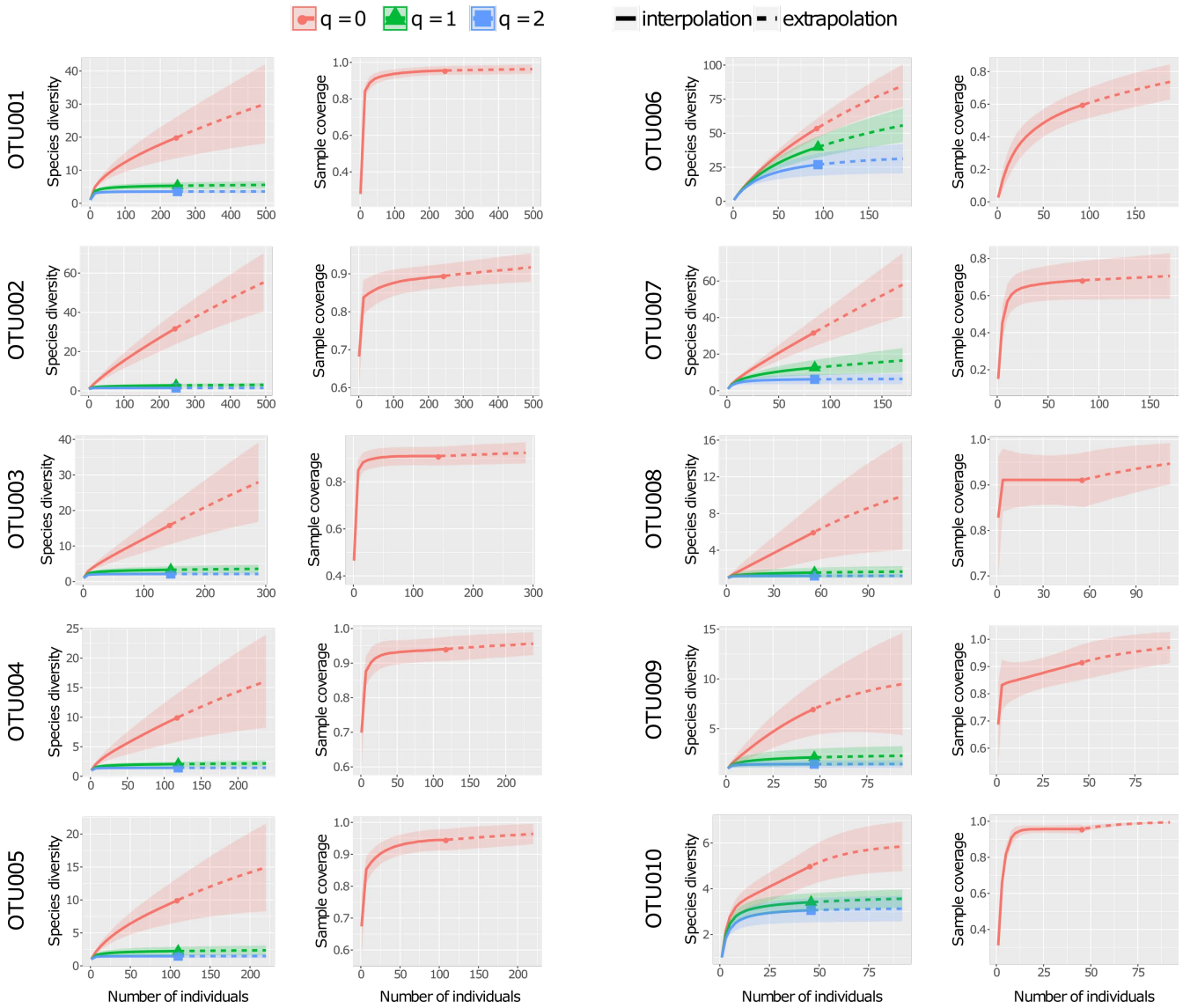


Figure S2. Box-and-whisker plot showing the latitudinal distribution of the main haplotypes of *Fusarium tricintum*, H1 and H3, respect to the overall distribution of the OTU.

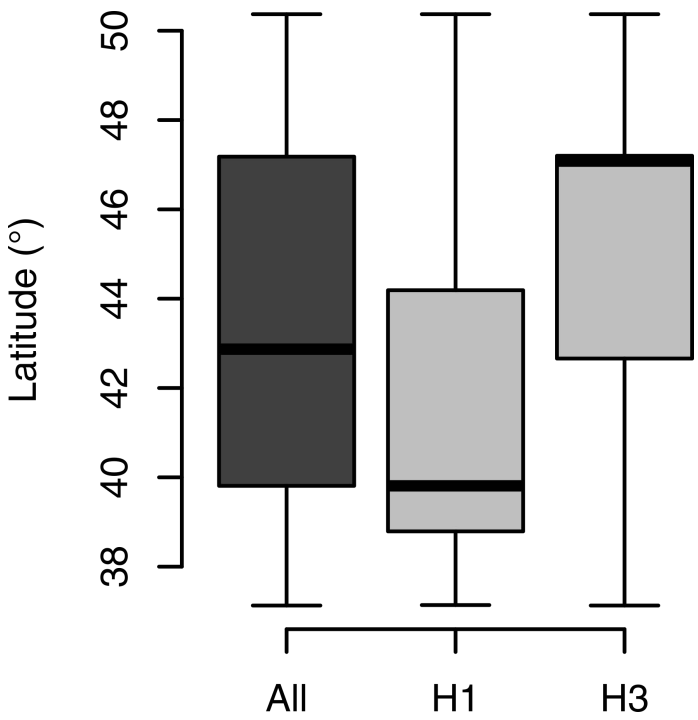
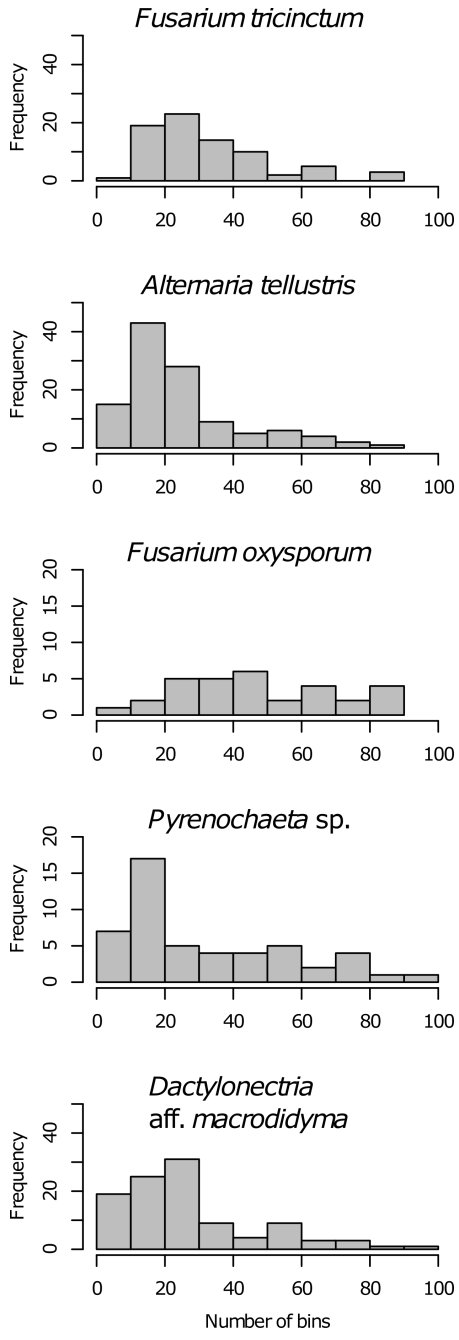


Figure S3. Distribution of AFLP bin frequencies for each OTU selected in this study.



Chapter 4: Kia SH, Glynou K, Nau T, Thines M, Piepenbring M, Maciá-Vicente JG. (2017). Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants. *ISME J* **11**: 777–790.

Anlage 3

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Co-Author SHK: 25%
Co-Author MT, MP: together 15%
Co-Author: JGMV: 60%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende KG: 10% isolation and molecular characterization of strains
Co-Author SHK: 70% plant-fungus co-inoculation assays, measuring of fungal traits, microscopical analyses.
Co-Author TN: 5% isolation and molecular characterization of strains.
Co-Author JGMV: 15% plant-fungus co-inoculation assays, measuring of fungal traits, microscopical analyses.

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende KG: 5% collection of sequencing data
Co-Author SHK: 80% collection of bioassays and fungal trait data
Co-Author TN: 5% collection of sequencing data
Co-Author JGMV: 10% collection of bioassays and fungal trait data

(4) zur Analyse und Interpretation der Daten

Co-Author SHK: 50%
Co-Author JGMV: 45%
Promovierende KG, MP, MT: together 5%

(5) zum Verfassen des Manuskripts

SHK: 50%
Co-Author JGMV: 40%
Co-Authors KG, MP, MT, TN: together 10%

Zustimmende Bestätigungen der oben genannten Angaben:

_____	_____
Datum/Ort	Unterschrift Promovend
_____	_____
Datum/Ort	Unterschrift Betreuer
_____	_____
Datum/Ort	Ggfs. Unterschrift *corresponding author*

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ORIGINAL ARTICLE

Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants

Sevda Haghi Kia[1,2], Kyriaki Glynou[1,2], Thomas Nau[1], Marco Thines[1,2,3], Meike Piepenbring[1,2] and Jose G Maciá-Vicente[1,2]

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Plants associate through their roots with fungal assemblages that impact their abundance and productivity. Non-mycorrhizal endophytes constitute an important component of such fungal diversity, but their implication in ecosystem processes is little known. Using a selection of 128 root-endophytic strains, we defined functional groups based on their traits and plant interactions with potential to predict community assembly and symbiotic association processes. *In vitro* tests of the strains' interactions with *Arabidopsis thaliana, Microthlaspi erraticum* and *Hordeum vulgare* showed a net negative effect of fungal colonization on plant growth. The effects partly depended on the phylogenetic affiliation of strains, but also varied considerably depending on the plant-strain combination. The variation was partly explained by fungal traits shared by different lineages, like growth rates or melanization. The origin of strains also affected their symbioses, with endophytes isolated from *Microthlaspi* spp. populations being more detrimental to *M. erraticum* than strains from other sources. Our findings suggest that plant–endophyte associations are subject to local processes of selection, in which particular combinations of symbionts are favored across landscapes. We also show that different common endophytic taxa have differential sets of traits found to affect interactions, hinting to a functional complementarity that can explain their frequent co-existence in natural communities.

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Introduction

Root endosymbiotic fungi have impacts on ecosystem functioning through their effects on plant productivity and community assembly (Bever *et al.*, 2010, 2012). Some, such as mycorrhizal fungi, develop mutualistic interactions that allow plants to exploit habitats that would otherwise be inaccessible to them, and to boost their competitiveness over plants lacking these associations (Bever *et al.*, 2010). Others have evolved pathogenic lifestyles and can reduce considerably their hosts' fitness, thus contributing to the diversity of plant communities (Van der Putten *et al.*, 1993; Wardle *et al.*, 2004; Mangan *et al.*, 2010). In addition to these relatively well-defined symbionts, healthy plant roots harbor a broad diversity of other fungi, referred to as root endophytes (Rodriguez *et al.*, 2009; Sieber and Grünig, 2013). The effects of root endophytes on their hosts' development are poorly known, and hence their function in natural ecosystems remains cryptic (Mandyam and Jumpponen, 2005).

Non-mycorrhizal endophytes represent the largest fraction of the fungal diversity within roots, and they are found in all plants and land ecosystems (Vandenkoornhuyse *et al.*, 2002; Sieber and Grünig, 2013). They form polyphyletic ensembles seemingly adapted to the root environment, as their structure and composition differ from those in the neighboring soil and plant organs (Maciá-Vicente *et al.*, 2012; Coleman-Derr *et al.*, 2016). Because the endophyte concept constitutes a catchall classification encompassing all symbionts in the interior of healthy plant tissues (Rodriguez *et al.*, 2009), it is likely to lump together fungal lineages with heterogeneous ecological roles. For example, it is argued that endophytes develop symbioses ranging from parasitic to mutualistic (Mandyam and Jumpponen, 2015) or that depend on the trade-off of particular resources (Newsham, 2011). Moreover, the occurrence of particular endophytes depends on host identity and environmental conditions (for example, Maciá-

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Vicente *et al.*, 2008a, 2012; Wehner *et al.*, 2014; Glynou *et al.*, 2016), they can occupy different root compartments, or follow distinctive patterns of colonization (Maciá-Vicente *et al.*, 2008b, 2009a, 2012; Peterson *et al.*, 2008; Atsatt and Whiteside, 2014). A comprehensive characterization of the symbiotic roles played by endophytes is necessary to understand the evolutionary processes determining the plant-associated fungal diversity and its contribution to the feedbacks that sustain natural communities (Bever *et al.*, 2012).

Many studies have aimed to assess the natural function of endophytes by reproducing their interaction with plants under controlled conditions (for example, Usuki and Narisawa, 2007; Maciá-Vicente *et al.*, 2008b, 2009a,b; Tellenbach *et al.*, 2011; Keim *et al.*, 2014; Mandyam and Jumpponen, 2014, 2015). They provide examples of specific associations between particular fungal and plant genotypes, but they are difficult to extrapolate to general scenarios owing to a high intra-specific variability of the interactions (Tellenbach *et al.*, 2011; Mayerhofer *et al.*, 2012) and to the difficulty in detecting responses in either symbiont (Mandyam and Jumpponen, 2005). Alternative approaches based on the measurement of fungal traits have been proposed to unravel the implication of fungi in ecosystem dynamics (Aguilar-Trigueros *et al.*, 2014, 2015). Classifications of species based on their sets of traits have been used to define major life history strategies, which, in turn, can predict patterns of biodiversity, community assembly and natural associations (Chagnon *et al.*, 2013).

Trait-based approaches have proven valuable to identify relationships between life history and functional traits of arbuscular mycorrhizal fungi and their plant interactions (for exampl, Powell *et al.*, 2009; Maherali and Klironomos, 2012; Chagnon *et al.*, 2013). For example, differences across arbuscular mycorrhizal fungal lineages in rates of nutrient exchange with hosts, sporulation and biomass allocation to mycelial compartments, are linked with their association and interaction with particular plants, their biogeographic and successional patterns, and their community structure (Chagnon *et al.*, 2013). Distinctive traits have also been used to define groups of non-mycorrhizal endophytes, like in the so-called dark-septate endophytes (DSE; Jumpponen and Trappe, 1998). But how these traits are relevant for the symbiosis is seldom known, and systematic studies on the patterns of distribution and evolution of characters across endophytic lineages are lacking (Aguilar-Trigueros *et al.*, 2014).

In this study, we examine the influence of phylogeny and traits of root-endophytic fungi on their interaction with plants. We employ a collection of strains isolated from different plant species, geographical locations and habitats. Most of them originate from a screening of the non-mycorrhizal plant *Microthlaspi* spp. (Brassicaceae) across Europe (Glynou *et al.*, 2016), which harbored a broad diversity of endophytes. In it, a few endophytes with disparate phylogenetic affiliations, like *Fusarium* spp., *Alternaria* spp. and *Cadophora* spp., were ubiquitous and co-existed frequently in the same root communities, but displayed distinctive distribution patterns and ecological preferences. Therefore, our collection provides a basis to assess patterns of trait variation across fungal lineages, geography and ecological conditions. Here, we measure life history traits of endophytes, such as growth rates and sporulation capacity, as well as traits proposed to be potentially functional for the symbiosis, like hyphal melanization and production of intraradical microsclerotia—defining characters of DSE—and enzymatic activities that can facilitate host nutrient uptake or assist fungal penetration of plant tissues (Mandyam *et al.*, 2010). In addition, we assess the effect of strains on the growth of *Microthlaspi erraticum*, its confamilial *Arabidopsis thaliana* and the gramineous *Hordeum vulgare* (Poaceae). Our aim is to test how the interactions between root-endophytic fungi and plants are influenced by phylogenetic conservatism, as well as by convergent traits and ecological origins of fungi that are unrelated to phylogeny.

Materials and methods

Fungal strains and plant material

One hundred and twenty-eight fungal strains isolated from roots of different plant species and geographical locations were used in this study. The majority originate from *Microthlaspi* spp. (Glynou *et al.*, 2016), whereas others were isolated from *Salicornia* spp. (Amaranthaceae). Endophytes were isolated in culture after the surface-sterilization of roots as described by Maciá-Vicente *et al.* (2012), and selected prior to their identification by choosing morphologically divergent strains from different plants/locations. In addition, we obtained *Serendipita indica* (syn: *Piriformospora indica*) CBS 125645 from the KNAW-CBS Fungal Biodiversity Centre. *S. indica* has been thoroughly studied as a model endophyte with a mutualistic interaction with multiple plants (Banhara *et al.*, 2015). A description of all strains is provided in Supplementary Table S1.

The plants *A. thaliana* ecotype Col-0, *M. erraticum* and *H. vulgare* cv. Barke (barley) were used as hosts in plant–endophyte interaction assays. Seeds of *A. thaliana* were provided by the Laboratory of Plant Physiology of Wageningen University. Seeds of *M. erraticum* were collected from a field population in Germany (Mp_K11; Ali *et al.*, 2016). Barley seeds were provided by the company Saatzucht Josef Breun GmbH & Co. KG (Herzogenaurach, Germany).

Molecular characterization of strains

We obtained the sequences of the ribosomal DNA internal transcribed spacer regions (ITS) of all

strains. ITS sequences from most strains were already available from Glynou *et al.* (2016), and the rest were obtained as described therein. We also followed the procedures in Glynou *et al.* (2016) to assign the strains to taxa and to group them into operational taxonomic units (OTUs). In brief, genomic DNA was extracted from fungal mycelia using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA). ITS sequences were amplified and sequenced using the primer pair ITS1F/ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993), and they were then classified at different taxonomic precisions with the Naive Bayesian classifier tool of Mothur v1.34.4 (Wang *et al.* 2007; Schloss *et al.* 2009), based on comparisons with the UNITE database of curated fungal ITS sequences (Kõljalg *et al.*, 2013). Strains were grouped into OTUs according to ITS pairwise similarity of at least 97%, using the BLASTClust program (Altschul *et al.*, 1997). The taxonomic classification of strains and the GenBank accession numbers of all sequences are provided in Supplementary Table S1.

We built a molecular phylogeny with the ITS sequences using Bayesian inference. The ITS1, 5.8S and ITS2 regions were independently aligned using MAFFT v7.123b (Katoh and Standley, 2013), and ambiguously aligned regions were removed using Gblocks v0.91b (Castresana, 2000). Two parallel MCMC analyses, using the GTRGAMMA model with independent parameter estimates for each partition, were run in MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001) for 10 M generations with sampling every 100th generation and 30% burn-in. An ultrametric majority-rule consensus tree was used in subsequent analyses. Whereas the ITS regions are not suitable for phylogenies involving distantly related taxa owing to their variability, our trimmed alignment consisted mostly of the conserved 5.8S gene. The latter has been used in phylogenies of highly divergent taxa (Redecker *et al.*, 1999), and our resulting tree reflected the OTU relationships among strains (Supplementary Figure S2).

Morphological and physiological characterization of strains

The strains were maintained in triplicate cultures on corn meal agar (CMA, Sigma-Aldrich, St. Louis, MO, USA) and malt extract agar (MEA, Applichem, Darmstadt, Germany). We recorded the presence/absence of conidia and darkly pigmented (dematiaceous) mycelia in cultures for up to 3 months. Radial growth rates were measured three days after plating on each medium and reported as millimeters of colony expansion per day. We measured the production of extracellular enzymes using custom plate assays. Cellulase activity was assessed by the clearing halo produced by 7-day-old colonies on Czapek-Dox agar with 0.5% (w/v) carboxymethylcellulose sodium salt as sole carbon source (Johnsen and Krause, 2014). Peptidase, pectinase, laccase and peroxidase activities were measured following the methods described by Basiewicz *et al.* (2012). In addition, the ability of strains to solubilize mineral phosphate was measured as in Zavala-Gonzalez *et al.* (2015). Cellulase, pectinase and phosphate solubilization activities were measured as the proportional width of the clearing halo respect to colony diameter. Peptidase, laccase and peroxidase activities were rated according to a 1–4 semi-quantitative scale.

Arabidopsis and microthlaspi inoculation assays

We tested the effect of colonization by individual strains on the development of *A. thaliana* and *M. erraticum* using an *in vitro* assay (Supplementary Figure S3). Surface-sterilized seeds were plated on half-strength Murashige-Skoog basal salt solid medium (MS, Sigma-Aldrich; Murashige and Skoog, 1962), stratified for 2 days at 4 °C in the dark, and then incubated for 7 days at 23 °C under continuous illumination (80 $\mu mol\ m^{-1}\ s^{-1}$). Upon emergence of the first true leaves, seedlings were transferred to 24-well plates containing MS medium and maintained in the same incubation conditions. After 10 days, plants were inoculated with individual strains or left uninoculated in controls. Because many strains did not sporulate in culture, inoculation was performed by puncturing the margin of actively growing colonies on CMA with a sterilized toothpick to collect a small amount of mycelium, and then transferring it a few millimeters from the crown of plants by inserting it in the agar. Ten days after inoculation, the development of mycelium in roots was confirmed under a stereomicroscope. Symptoms of chlorosis and/or necrosis in leaves were rated on a semi-quantitative scale (0 = none, 1 = up to 30% chlorotic/necrotic leaves, 2 = 30–60%, 3 = > 60%, 4 = dead plants), and the fresh weight of the aerial tissues was measured. Every treatment consisted of five replicates, performed simultaneously in a separate 24-well plate each. The layout of treatments was randomized within wells to minimize potential effects on the data owing to position. Experiments were performed in batches including 23 strains and a control treatment each, and measurements for each fungal treatment were compared only to its respective control. To assess the reproducibility of assays, we repeated them for 34 strains in *A. thaliana* (Supplementary Figure S4).

Barley inoculation assays

The effects of root colonization on barley were assessed using a standard *in vitro* assay (Dufresne and Osbourn, 2001; Maciá-Vicente *et al.*, 2008b). In brief, 2-day-old seedlings obtained from

surface-sterilized seeds were planted in glass tubes with 30 ml sterilized, hydrated vermiculite. Four 5-mm-diameter plugs taken from the margin of actively growing colonies on CMA were used as inoculum, by placing them 2–3 cm deep in the vermiculite. Control tubes were mock-inoculated with sterile CMA plugs. Plants were grown under long day conditions (16 h:8 h, light:dark, 80 μmol m^{-1} s^{-1}) at 23 °C. The fresh weight of roots and shoots was measured after 10 days. In this case, we did not score symptoms because they were seldom evident in leaves, and detrimental effects were manifested by reductions on biomass (Supplementary Figure S3). Treatments consisted of 10 replicates, which were performed in batches of three to nine strains and one un-inoculated control treatment each. We repeated these assays for five strains to test their reproducibility (Supplementary Figure S4).

We assessed the endophytic colonization of roots by cultivation and microscopy methods. In the first case, one to two roots per plant were surface-sterilized for 1 min with 0.5% sodium hypochlorite, rinsed with sterilized deionized water, and cut into 0.5 cm pieces. Ten root pieces per plant were randomly selected, dry-blotted onto sterilized paper and plated on CMA. The efficacy of the sterilization procedure was assessed in a subset of 30 root pieces per treatment with the imprint method (Hallmann *et al.*, 2006). The percentage of root pieces colonized was recorded 5–7 days later. For the microscopical observation of root colonization, we randomly selected three barley plants per treatment. One entire seminal root per plant was cleared overnight in a 1 M KOH solution, stained with acidified lactophenol blue and kept in acidified glycerol until observation. Samples were observed in squash preparations, in which epiphytic and/or endophytic root colonization, and the presence of microsclerotia were recorded (Supplementary Figure S5). The latter was considered as a fungal trait in subsequent analyses.

Statistical analyses

Data organization. Statistical analyses were performed using R v3.0.2 (R Core Team, 2013). Data from the inoculation assays were first assessed for normality and homoscedasticity, and then treatments were compared using analysis of variance or the Kruskal–Wallis test. Subsequent pairwise comparisons of each fungal treatment against its respective control were done by either *t*-tests or Wilcoxon tests with a Holm–Bonferroni correction. In order to incorporate these data into further analyses, we calculated the effect size of biomass variables from each treatment respect to its un-inoculated control. Effect sizes with 95% confidence intervals were calculated according to the Cohend's *d* statistic (Cohen, 1988) using function *cohen.d* in package *effsize* v0.5.4 (Torchiano, 2015), which measures the difference in means and standardizes it by their pooled s.d.

Fungal identifications resulted in several strains isolated from the same plant population being assigned to the same OTU. We considered them likely to belong to the same genets. In order to avoid repeated observations that could inflate the significance of tests, we thinned our datasets to 115 strains representing unique potential genets (Supplementary Figure S2). Pairs of strains within each potential genet often showed similar effects on plant growth (Supplementary Figure S6), and hence alternative selections of strains hardly changed results in downstream analyses.

Analysis of fungal traits. We used principal component analysis with standardized morphological and physiological trait data to summarize the differences among the 115 selected strains. We assessed the goodness of fit of the principal component analysis using the broken-stick criterion, which tests the cumulative percentage of variance explained respect to a random breakdown of variance. Individual variables significantly contributing to axes were identified using the equilibrium circle method (Legendre and Legendre, 2012).

Measurement of phylogenetic signal. We calculated the phylogenetic signal in the response of plant growth to fungal inoculation with the K statistic (Blomberg *et al.*, 2003), using function *phylosig* in package *phytools* v0.5 (Revell, 2011). The method is used to assess conservation of traits among species, with $K=0$ indicating absence of a phylogenetic signal, and $K<1$ or $K>1$ resemblance lower or higher than expected under Brownian motion evolution. Because simultaneous inferences of phylogenetic signal between species and within species are difficult to interpret (Blomberg *et al.*, 2003), we only included in these analyses individual values for each OTU as the mean of the effects by its strains. Sampling errors of within-OTU variability were incorporated following Ives *et al.* (2007), assuming variances for OTUs with only one strain equal to the mean of the overall within-OTU variance. The significance of K was assessed by comparing with a random shuffle of values at the tips of the phylogenetic tree. We also tested for phylogenetic signal in the interactions of strains within the orders Pleosporales, Hypocreales or Helotiales, separately.

Contribution of strain features to plant interactions. To assess the influence of phylogeny, traits and origin of strains on plant growth, we applied the variation partitioning method described by Desdevises *et al.* (2003) with function *varpart* in package *vegan* v2.2–1 (Oksanen *et al.*, 2015). We performed the tests independently for each plant species, including as a response variable the effect size of their biomass as affected by every fungus. The

variation was decomposed into three independent explanatory matrices gathering variables related to the strains' traits, origin (geographical coordinates, and natural host as *Microthlaspi* spp. or others) and phylogeny (principal coordinates (PCs) obtained from the phylogenetic tree). We retained only 19 PCs that were significantly correlated in linear regressions ($P<0.05$) with the effects on at least one plant, representing a combination of early (PCs 1 and 2) and late phylogenetic divergences. The significance of the variance fractions explained by each component was tested using permutation tests with pseudo *F*-ratios.

To estimate the contribution of each fungal lineage to tree-wide variation in traits, we calculated their contribution indices (Moles *et al*., 2005) using the *aotf* function in the program *phylocom* v4.2 (Webb *et al*., 2008). The index measures the proportional contribution of individual nodal divergences along the phylogeny to extant trait variation. Statistical support is assessed by comparing the values with those obtained by a random shuffle of traits at the tree tips. A trait can be considered conserved if more variation is explained by ancient than by recent divergences (Maherali and Klironomos, 2012).

We tested for potential relationships between individual trait/origin variables and the effects on plant biomass, using phylogenetic generalized least squares to account for phylogenetic signal in the data. Phylogenetic generalized least squares estimates regression parameters weighted by phylogenetic signal measured as Pagel's λ (with 0 and 1 indicating no or strong signal, respectively; Pagel, 1999), and it is equivalent to an ordinary least squares model when the signal is absent in the residuals (Symonds and Blomberg, 2014). These analyses were carried out using function *pgls* in package *caper* v0.5.2 (Orme *et al*., 2011).

Results

Taxonomic classification of strains

The strains were classified in 54 OTUs and ascribed to 17 families in 11 orders. Among the 115 strains representing likely independent genets, 111 (96.5%) were species of *Ascomycota* and four (3.5%) of *Basidiomycota*. The most frequently encountered orders were Pleosporales and Hypocreales, with 56 (48.7%) and 39 (34%) strains belonging to 26 (48.1%) and 11 (20.4%) OTUs, respectively (Supplementary Figure S1). They were followed by Helotiales, with 10 strains (8.7%) in seven OTUs (13%), whereas other orders were represented by two or less strains each (Supplementary Figure S1). Within Pleosporales, 29 strains (25.2%) in seven OTUs (13%) belonged to the family Pleosporaceae and were mainly represented by OTUs related to *Alternaria* spp. Other OTUs within Pleosporales were designated as family *incertae sedis* or remain unclassified. Most members of Hypocreales belonged to Nectriaceae, with 31 strains (27%) out of which 29 belonged to six OTUs classified as *Fusarium* spp. The most frequent of these were OTU001 with affinities to *Fusarium tricintum* and *Fusarium avenaceum*, and OTU003 related to *Fusarium oxysporum*. Other species of Hypocreales were assigned to *Emericellopsis* and *Ilyonectria*, with families *incertae sedis*. Six Helotiales strains (5.2%) had ITS affinities with the genus *Cadophora* with family *incertae sedis*, and are referred to as *Cadophora*-like onwards (Supplementary Figure S1).

Characterization of strain traits

We measured variables of morphology, growth rates and enzymatic and phosphate solubilization activities in all fungal strains (Supplementary Table S1; Supplementary Figure S5). A principal component analysis ordination of the trait data explained an overall 48.4% of the variance (Figure 1), larger than that explained by a random breakdown of variance (36%). The first component collected most information related to the strains' growth rates and clearing halos (Figure 1a). The second component mainly represented variation in peroxidase activity, pigmentation and production of intraradical microsclerotia (Figure 1a). The ordination of strains reflected their phylogenetic affinities (Figure 1b), with a clear separation of Hypocreales, Pleosporales and *Cadophora*-like strains along the first axis. Members of Nectriaceae formed a compact cluster clearly separated from other Hypocreales and most other fungi (Figure 1b). Strains within Pleosporaceae showed a tendency toward a high peroxidase activity and the formation of dematiaceous mycelia and microsclerotia, although they also showed a wide variability in these characters (Figure 1b).

Effect of fungal strains on plant growth

Inoculation assays of individual strains in *A. thaliana*, *M. erraticum* and *H. vulgare* yielded a wide range of growth responses, ranging from a strong inhibition to a moderate stimulation of plant biomass production in comparison to un-inoculated controls (Figure 2; Supplementary Figure S3). The overall effect of fungal inoculation was negative for all host species ($W=395$–1685, $P<0.001$), but it was less marked in barley. Similar results were obtained when considering variables of plant development other than total biomass, because they were strongly collinear with it (Pearson's $r<-0.74$, $P<0.001$ for symptoms data in both Brassicaceae; $r>0.97$, $P<0.001$ for the effects on shoot and root biomass in *H. vulgare*). Moreover, similar effects were observed in repetitions of these experiments with subsets of strains (Supplementary Figure S4).

We only found conservatism in the response of *M. erraticum* to fungal inoculation (Table 1). OTUs within the Hypocreales had a conserved effect on

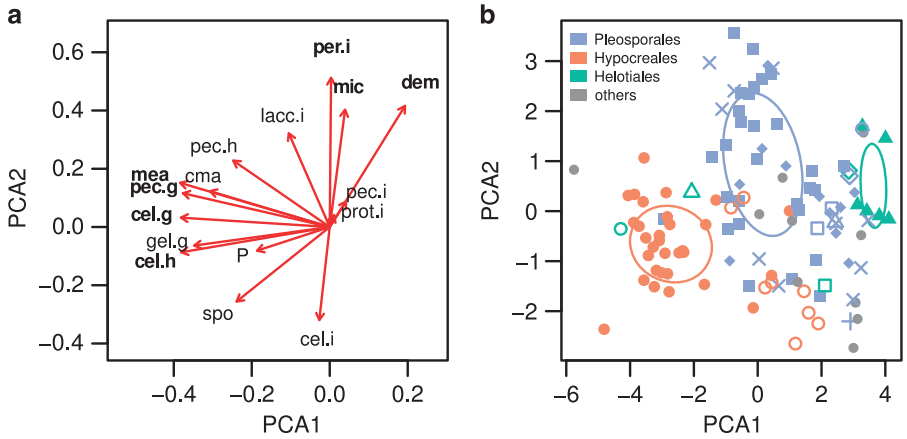

Figure 1 Principal component analysis (PCA) ordination of fungal endophytic strains according to their physiological and morphological traits. The two axes represent 36.2% and 12.2% of the data variance, respectively. (**a**) PCA scores showing the contribution of each trait to the separation of the strains, as indicated by the direction and magnitude of the respective arrows. Variables in bold contributed significantly to the variance, according to the equilibrium circle method. (**b**) Ordination of strains according to their traits. Strains belonging to the three most represented fungal orders are shown in different colors (see color key). Different symbols within each of these orders indicate strains belonging to different families, or to paraphyletic groups at that taxonomic level. Ellipses delimit 95% confidence intervals around the strains of Nectriaceae (solid circles), Pleosporaceae (solid squares) and Helotiales *incertae sedis* (=*Cadophora*-like, solid triangles). Abbreviations: *cel.g*, growth rate on cellulose; *cel.h*, degradation halo on cellulose; *cel.i*, cellulase activity; *cma*, growth rate on CMA; *dem*, pigmentation; *gel.g*, growth rate on gelatin; *lacc.i*, laccase activity; *mea*, growth rate on MEA; *mic*, production of microsclerotia; *P*, phosphorus solubilization; *pec.g*, growth rate on pectin; *pec.h*, degradation halo on pectin; *pec.i*, pectinase activity; *per.i*, peroxidase activity; *prot.i*, protease activity; *spo*, production of conidia.

A. thaliana but non-significant signals in their interactions with *M. erraticum* and *H. vulgare* (Table 1). The responses to fungal inoculation varied considerably across plants and fungal lineages. *M. erraticum* and *H. vulgare* were most negatively affected by *Fusarium* spp. strains, whereas the strongest negative effects on growth of *A. thaliana* were caused by members of the Pleosporaceae (Figure 2, Supplementary Figure S7). Fungal OTUs with the strongest overall virulence towards either plant species, such as *Fusarium* spp. OTU001 and OTU003, and *Alternaria* sp. OTU008, also showed a broad within-group variability that spanned the entire range of interactions (Figure 2, Supplementary Figure S7). The effects of *Cadophora*-like strains were always close to neutrality (Figure 2, Supplementary Figure S7).

Fungal colonization of roots

Fungal root colonization was detected in most plants at the moment of sampling. In *H. vulgare*, we quantified the degree of colonization in culture, and we often observed it directly by light microscopy (Supplementary Figure S5). We did not find a significant phylogenetic conservatism in the fungal colonization of barley roots ($K=0.7$, $P=0.9$). Root colonization was negatively correlated, after controlling for phylogenetic signal, with the effect on total plant biomass of each strain (slope $=-0.023\pm0.003$, adj. $R^2=0.27$, $F_{124}=32.82$, $P<0.001$, $\lambda=0$). A similar result was obtained for the effect on shoot and root biomass (Supplementary Table S2). Moreover, root colonization was positively correlated with all variables of fungal growth ($P<0.01$).

Contribution of strain features to plant interactions

We evaluated the contribution of strain variables related to phylogeny, traits and origins, to the effect of fungal inoculation on plant growth (Figure 3). Models for each plant explained a significant proportion of the variation in their growth response to fungal inoculation (48–59%, $P<0.001$). The phylogeny of strains predicted the largest fraction of the variance in *A. thaliana* and *M. erraticum* (45.1 and 35.7%), but it was less informative than the strains' traits in *H. vulgare* (25.1% respect to 33%). Some fungal clades contributed greatly to the overall phylogenetic signal, mainly representing late divergences in the phylogenetic tree at the OTU level (Figure 4). *Fusarium* sp. OTU001 had the largest contribution to overall variance in the responses of *M. erraticum* and *H. vulgare*, whereas divergences in growth of *A. thaliana* were most affected by several pleosporaceous OTUs (Figure 4). These late divergences contrast with those obtained for mycelial traits, which tended to be greater earlier in the phylogeny (Figure 4, Supplementary Figure S8). The response of both Brassicaceae had little dependence on the strains' traits alone (4–8%), which had an effect partly indistinguishable from that of the strains' phylogeny in *M. erraticum* (Figure 3). Fungal traits explained a significant amount of the interactions with *H. vulgare* (33.1%). Only the response of *M. erraticum* to colonization was significantly correlated with the strains' origin (Figure 3).

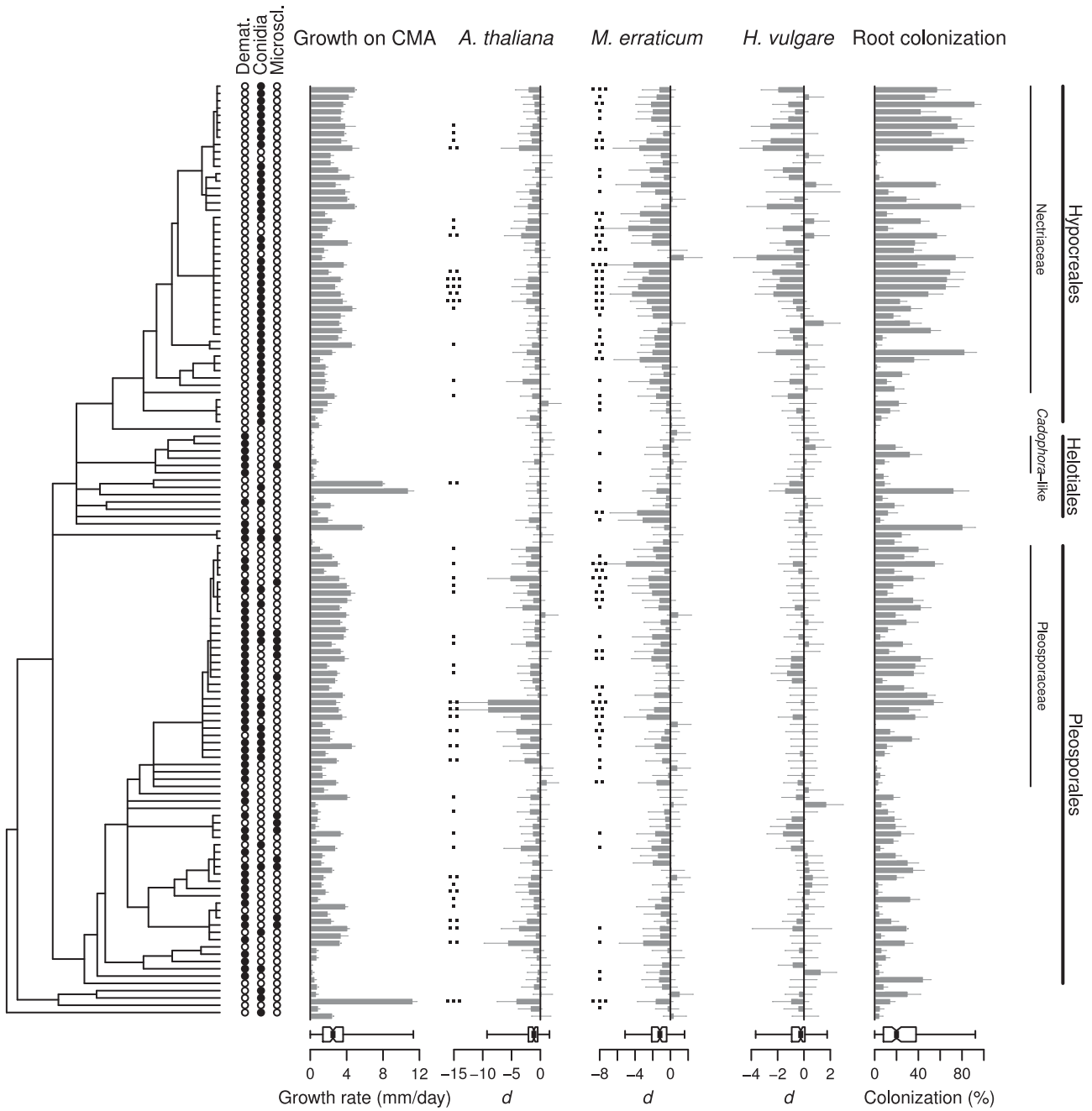

Figure 2 Interactions between 128 fungal endophytic strains and the plants *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*. Selected fungal traits are also shown. Bars represent effect sizes (Cohend's *d*) ± 95% confidence intervals for the interactions with plants, and mean values ± s.e. for the growth rates of strains on corn meal agar (CMA) and barley root colonization. Boxplots at the bottom of graphs represent the overall data distribution for each variable. Points next to bars for *A. thaliana* and *M. erraticum* indicate average scores >1 in a semi-quantitative scale of symptoms. Solid and empty bullets in the qualitative traits of dematiaceous mycelium (Demat.), production of conidia, and production of microsclerotia (Microscl.) indicate presence or absence of the character, respectively.

Phylogeny-independent determinants of plant–endophyte interactions

We assessed the correlation between individual strain variables included in the variation partition and the response of plants to fungal colonization using phylogenetic generalized least squares to subtract phylogenetic signal. All descriptors of hyphal growth were strongly associated with negative effects on the development of the three plants (Table 2). Of the physiological characteristics of strains, only laccase and pectinase activities showed a significant association with biomass of *H. vulgare* (Table 2). Production of dematiaceous mycelium and conidia had contrasting positive and negative relationships with the development of individual plant species, respectively (Table 2).

Table 1 Phylogenetic signal in the growth responses to fungal inoculation of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*, according to Blomberg's *K*.

Fungal group[a]	A. thaliana		M. erraticum		H. vulgare	
	K[b]	P	K	P	K	P
All fungi	0.8	0.8	**0.9**	**0.029**	0.7	0.5
Pleosporales	0.6	0.4	0.9	0.9	0.9	0.8
Hypocreales	**0.9**	**0.048**	0.2	0.7	0.2	0.7
Helotiales	0.9	0.5	1	0.8	0.6	0.7

[a]Phylogenetic signal was tested in the plant interactions with all strains, and with strains within Pleosporales, Hypocreales, or Helotiales alone.
[b]$K=0$ indicates random evolution of traits, and $K=1$ indicates trait evolution under Brownian motion.
Significant values of K ($P<0.05$) are shown in bold face.

Interestingly, strains originally isolated from *Microthlaspi* spp. showed a stronger virulence than strains from other sources toward the congeneric *M. erraticum* (Table 2).

Discussion

We provide evidence that the effects of non-mycorrhizal fungal root endophytes on plant growth are strongly influenced by the phylogeny of fungi. However, the phylogenetic signal is mostly explained by recent divergences that indicate little conservatism in the evolution of interactions. Moreover, particular fungal traits shared by phylogenetically dispersed taxa affected to a different extent the plant responses to fungal inoculation. These effects always followed a similar trend in different plant species, suggesting a direct relation of the traits with specific types of associations, or their linkage with other characters relevant to the symbiosis (Treseder and Lennon, 2015). The collation of these traits across strains allows a rough functional classification of the fungal diversity included in our study, and to hypothesize about their influence in the assembly of natural root-endophytic communities.

Effect of fungal colonization on plant development

The net effect of fungal colonization on plant biomass was negative, consistent with previous results based on the controlled inoculation of plants with root endophytes (Tellenbach *et al*., 2011; Mayerhofer *et al*., 2012; Keim *et al*., 2014; Mandyam and Jumpponen, 2015). Our experimental system included the plant as the sole carbon source to sustain fungal growth, which conditioned a strong negative correlation between fungal development and plant biomass, similar to that reported in other endophytic interactions (Tellenbach *et al*., 2011). However, the negative responses were often small, and strong compromises of plant growth and development of symptoms were scarce. Most root endophytes do not seem an important burden to their hosts, suggesting that their parasitism may be easily compensated by slight enhancements of plant fitness in their natural habitat. Mutualistic interactions depend on a balance between net costs and benefits provided by symbionts, whereby they can become parasitic in the absence of the ecological factors that drive the relationship. There are multiple instances of non-mycorrhizal endophytes providing their hosts with benefits when exposed to external factors, such as pathogens (Maciá-Vicente *et al*., 2008b), environmental stress (Rodriguez *et al*., 2008) or nutrient shortages (Usuki and Narisawa, 2007; Behie *et al*., 2012; Hiruma *et al*., 2016). Endophytes are likely implicated in different yet unknown context-dependent trade-offs associated with conditions not reproduced in our system. A blind testing of multiple environmental factors was out of the scope of our study, but further work in this direction may help to unravel context-dependent symbioses.

Phylogenetic conservatism of plant–endophyte interactions

Plant responses to endophytic colonization partly depended on the phylogenetic relations of strains, suggesting the evolution of distinctive strategies for the interaction with hosts. These effects varied markedly across plant species, in line with studies evidencing a large dependency of plant–fungus symbioses on the specific combination of partners (Klironomos, 2003; Mandyam and Jumpponen, 2015). Much of the variation was associated with negative feedbacks with fungi related to well-known pathogens, particularly species within *Fusarium*, and Pleosporaceae like *Alternaria* spp. These lineages often dominate roots of healthy wild plants (Maciá-Vicente *et al*., 2008a, 2012; Knapp *et al*., 2012; Sánchez Márquez *et al*., 2012; Glynou *et al*., 2016), where their detrimental effects appear to be mitigated by environmental conditions and/or interactions with extant microorganisms. But the linkage between particular responses and well-defined fungal clades was not clear-cut, because several strains in the most virulent groups had little impact on plant growth. It would be reasonable to expect a clear differentiation in the associations involving particular fungal lineages, given the diverse life histories that determine distinctive physiological and morphological adaptations. Such trends have been identified in endophytes (Mayerhofer *et al*., 2012), but they are generally diffuse owing to a high intraspecific variability that often exceeds the variation between species (Tellenbach, *et al.* 2011; Mandyam and Jumpponen, 2014). Fungal determinants of the symbiosis might be subject to rapid and recent change over evolutionary time, perhaps separating different genetic populations within species. For example, *Fusarium* species show a wide intraspecific variability in their virulence because they have pathogenicity genes subject to strong diversifying pressure or that can be horizontally transferred

with mobile chromosomes (Ma *et al*., 2010; Sperschneider *et al*., 2015). Further, Cheikh-Ali *et al*. (2015)) found that root endophytes of a same phylotype isolated from distant localities expressed divergent morphological and physiological characters. The high intra-specific variation of plant–

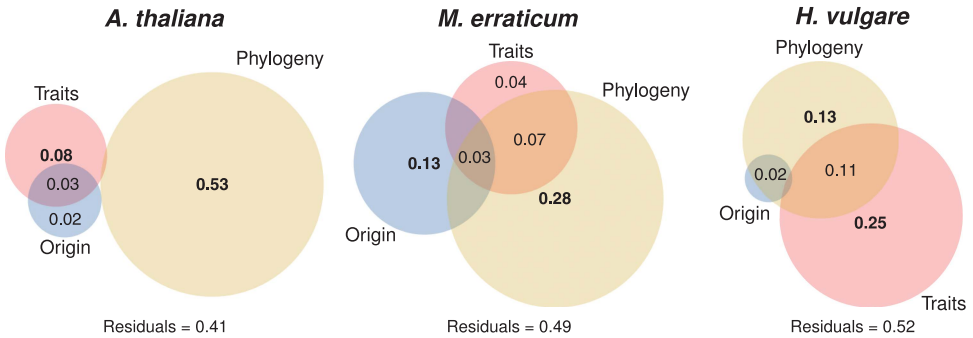

Figure 3 Euler diagrams of variation partitioning analysis, showing the effects of traits, origin and phylogeny of the endophytic strains on the growth of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*. Values indicate the proportion of the variation explained (adjusted R^2) by each fraction, corresponding to the pure effects of explanatory variables, or their shared effects (overlapping fractions). Values in bold are significant ($P<0.05$). Zero and negative values are not shown.

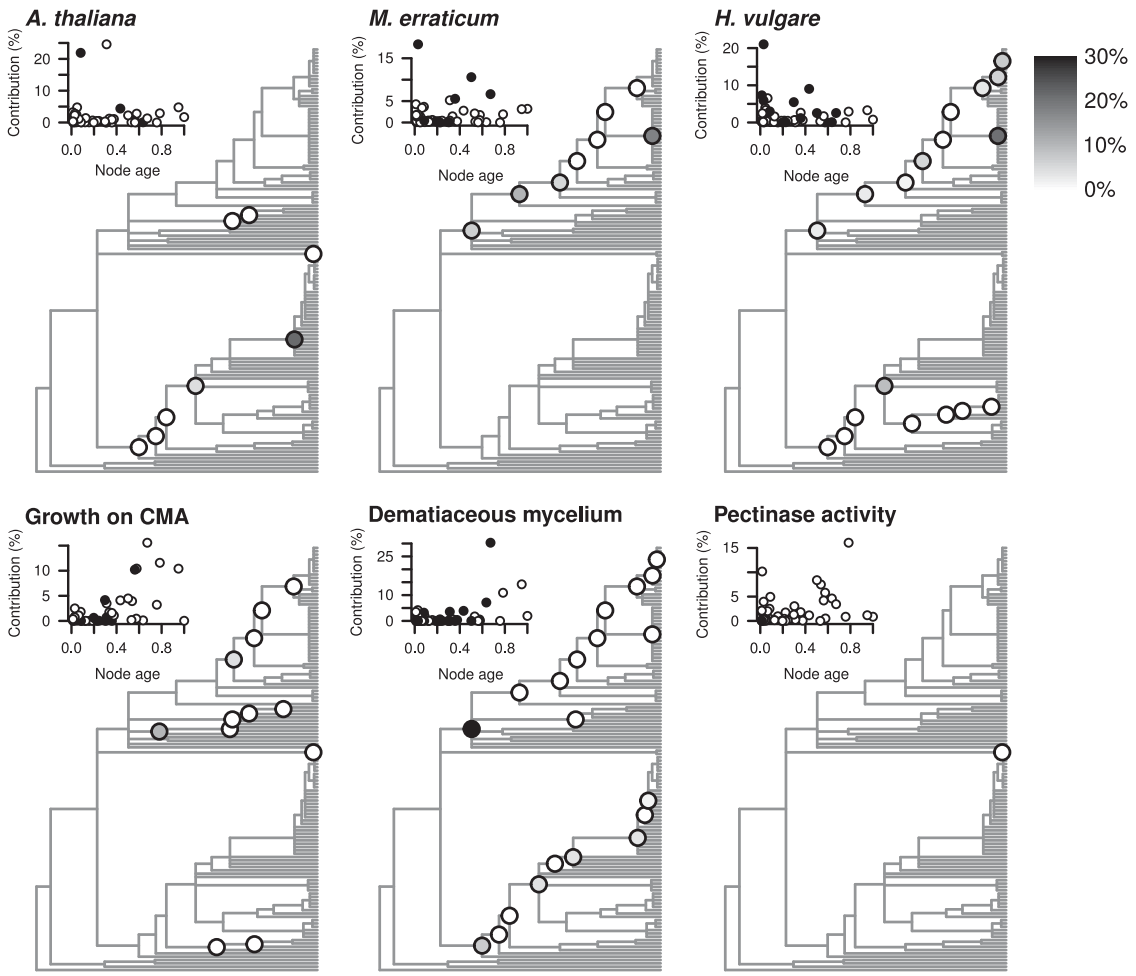

Figure 4 Nodal contributions to tree-wide variation in the response of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* to fungal inoculation, and to selected fungal traits relative to their morphology and physiology. Circles in the tree nodes indicate contribution indices that are significant ($P<0.05$), as compared with a distribution of 999 values calculated by a random shuffle of trait values across the tips of the phylogeny. Shading of circles represents the relative contribution of individual nodes to extant trait variation (see key). Scatterplots in insets show the relationship between the contribution indices and the respective age of nodes, with black and white circles indicating contribution indices that are significant or not, respectively.

Table 2 Phylogenetic generalized least squares regression models of the relations between plant growth responses to fungal inoculation, and variables of the strains' traits, geographical origin and natural host

Variable	A. thaliana[a]				M. erraticum				H. vulgare			
	Slope (± s.e.)	*Adj.* R^2	P-*value*	λ	*Slope (± s.e.)*	*Adj.* R^2	P-*value*	λ	*Slope (± s.e.)*	*Adj.* R^2	P-*value*	λ
Growth rate on corn meal agar	−0.24 ± 0.07	0.08	0.002	0	−0.18 ± 0.07	0.05	0.008	0.1	−0.16 ± 0.04	0.09	0.001	0.1
Growth rate on malt extract agar	−0.29 ± 0.08	0.09	<0.001	0.1	−0.29 ± 0.07	0.14	<0.001	0	−0.25 ± 0.04	0.21	<0.001	0
Growth rate on cellulose	−0.03 ± 0.01	0.15	<0.001	0.1	−0.02 ± 0	0.2	<0.001	0	−0.02 ± 0	0.21	<0.001	0
Growth rate on gelatin	−0.4 ± 0.13	0.07	0.003	0.1	−0.38 ± 0.11	0.09	0.001	0	−0.4 ± 0.07	0.22	<0.001	0
Growth rate on pectin	−0.37 ± 0.09	0.12	<0.001	0.1	−0.29 ± 0.08	0.11	<0.001	0	−0.27 ± 0.05	0.18	<0.001	0
Cellulase activity	—	—	—	—	—	—	—	—	—	—	—	—
Protease activity	—	—	—	—	—	—	—	—	—	—	—	—
Laccase activity	—	—	—	—	—	—	—	—	−0.17 ± 0.07	0.04	0.022	0.2
Pectinase activity	—	—	—	—	—	—	—	—	0.36 ± 0.16	0.04	0.022	0.2
Peroxidase activity	—	—	—	—	—	—	—	—	—	—	—	—
Phosphorus solubilization	—	—	—	—	—	—	—	—	—	—	—	—
Production of conidia	−0.64 ± 0.31	0.03	0.042	0.1	−0.53 ± 0.24	0.03	0.03	0	—	—	—	—
Production of microsclerotia	—	—	—	—	—	—	—	—	—	—	—	—
Pigmentation	—	—	—	—	0.62 ± 0.24	0.05	0.011	0	0.59 ± 0.17	0.09	0.001	0
Geographic latitude	—	—	—	—	—	—	—	—	—	—	—	—
Geographic longitude	—	—	—	—	—	—	—	—	—	—	—	—
Natural host (*Microthlaspi* vs others)	—	—	—	—	−1.11 ± 0.3	0.1	<0.001	0.1	—	—	—	—

Only model data of variables with a significant effect ($P<0.05$) are shown.
[a]Results of PGLS models show the slope (± s.e.) of the fitted line representing the correlation between variables, the coefficient of determination, the *P*-value of the model, and the estimate of the phylogenetic signal associated with the regression as Pagel's λ.

endophyte interactions has likely ecological implications for the local assembly of natural communities, because it might be a consequence of adaptations to local conditions. This would promote selection mosaics across landscapes in which particular combinations of symbionts are favored by their joint response to extant conditions (Thompson, 2005; Piculell *et al*., 2008).

The broad variability in plant–endophyte interactions contrasts with patterns of evolution in functional traits of arbuscular mycorrhizal fungi, which appear to be phylogenetically conserved at the family level (Powell *et al*., 2009; Chagnon *et al*., 2013). The opposing patterns between mycorrhizal and non-mycorrhizal fungal endosymbionts probably reflect large differences in their specialization for the symbiosis. Unlike mycorrhizas, most endophytes are not bound to their plant hosts and can be found as saprotrophs in other substrata. Therefore, their evolution might be less subjected to constraints imposed by the symbiotic lifestyle.

Phylogeny-independent determinants of plant–endophyte interactions

The growth responses to fungal colonization varied among plant species. Interactions involving either brassicaceous host had a great dependency on the phylogenetic relations of strains, whereas in *H. vulgare* convergent fungal traits were more important in explaining the growth responses to inoculation. The distinctive results across plants could have been determined by methodological differences in the bioassays. Nevertheless, *A. thaliana* and *M. erraticum* were tested using a similar setup yet they had divergent responses to particular fungal groups like fusaria or Pleosporaceae strains, whereas growth patterns of *M. erraticum* had commonalities with those of barley. Besides, only *M. erraticum* showed a significant degree of conservatism in its response to close fungal relatives, which can be indicative of mutual adaptations between partners, given the affiliation of the plant with the natural host of most strains. Therefore, it seems likely that the variation in individual plant–endophyte combinations largely reflects actual specificities across partners.

In all cases, a significant amount of the variation was explained by traits shared by dispersed fungal clades, which were correlated with the outcome of interactions in a similar manner. Among these, the growth rates of strains were strongly associated with reductions of plant biomass, probably owing to the tendency of these strains to colonize host tissues systemically and to their larger demands on plant carbon. Likewise, strains capable to sporulate in culture tended to be more detrimental to plants, perhaps owing to the linkage of this trait with fast rates of mycelial growth.

Dematiaceous fungi were less prone to develop detrimental symbioses. Melanized hyphae, in combination with lack of spores in culture and the production of intraradical microsclerotia are defining attributes of DSE. These form a polyphyletic

group of fungi frequently regarded as potential mutualists based on their high prevalence and ubiquity in roots (Mandyam and Jumpponen, 2005), although their symbiotic function is still elusive. Newsham (2011) detected a net positive effect of DSE on plant performance, associated with increments in nutrients uptake in the presence of soil organic matter. The hydrolytic capabilities of several DSE have been previously described and suggest that they are able to access detrital nutrient pools as saprotrophs (Caldwell *et al*., 2000; Mandyam and Jumpponen, 2005; Mandyam *et al*., 2010). Our inoculation assays did not include organic sources of nutrients available for the fungi other than the plant, but the saprotrophic capabilities of DSE might entail in nature a fitness benefit to hosts that could easily overcome their weak parasitism.

We were unable to detect direct substantial effects of the strains' physiological activities on the outcome of interactions. Laccase and pectinase activities were the only traits somewhat associated with plant performance in barley. The expression of hydrolytic activities was highly variable in our assays, perhaps reflecting different substrate specificities and inducing conditions (Basiewicz *et al*., 2012), or unspecificities in the detection of particular activities (Johnsen and Krause, 2014). But this variation also highlights large differences among and within fungal taxa that suggest a broad diversity of potential interactions in response to the availability of substrates. This could be ultimately confirmed by comparing genomic traits relative to these activities and the assessment of their expression *in planta* (Lahrmann *et al*., 2015).

The original host of strains had a strong impact on their interactions with *M. erraticum*, in which strains isolated from congeneric plants were more virulent. This could indicate a certain host specificity of these strains that is backed by their phylogenetically conserved effect on this plant. Similar effects have been described for other root-endophytic (Tellenbach *et al*., 2011), pathogenic (Sacristán and García-Arenal, 2008) and mycorrhizal symbioses (Klironomos, 2003; Hoeksema and Thompson, 2007), what supports the hypothesis of symbiotic partners co-evolving in response to each other. Interestingly, stronger adaptations of root endophytes to their hosts often lead to an increased virulence (Tellenbach *et al*., 2011), as opposed to those involving mutualistic mycorrhizas (Hoeksema and Thompson, 2007). This hints to parasitism as the main lifestyle adopted by many root endophytes in nature.

Trait-based classification of strains

The grouping of strains based on the similarity of their traits clearly separated endophytic lineages that frequently dominate and co-exist in roots, particularly those related to *Fusarium*, Pleosporaceae and *Cadophora*-like (Glynou *et al*., 2016). The clustering was influenced by life history traits associated with the plant's response to infection, suggesting a differential niche occupancy by groups of endophytes likely to condition their spatial distribution (Violle *et al*., 2007; Violle and Jiang, 2009). *Fusarium* spp. clearly differed from other taxa by fast growth and production of conidia. These characters are associated with an efficient ability of dispersal and resource colonization, which is consistent with the broad distribution across Europe observed for OTUs in this group (Glynou *et al*., 2016). Pleosporaceous and *Cadophora*-like strains, on the other hand, had slower growth rates and exhibited traits typical of DSE, and their geographical distribution apparently is constrained by environmental factors such as climatic and soil variables (Glynou *et al*., 2016). Spatial distribution is often used as a proxy of niche breadth, but this principle has been shown to be less applicable to microbes than to macroorganisms (Carbonero *et al*., 2014). Microorganisms highly specialized for a particular factor can have broad distributions if the latter is widespread, because they are less affected by other conditions than other generalist species. Consequently, the wide spatial occurrence of *Fusarium* spp. independently of other environmental factors could be a result of their efficient adaptations to colonize roots.

Fungal traits as determinants of community assembly

Our selection of strains represents well the composition and structure of endophytic assemblages associated with *Microthlaspi* spp. and other plants (Maciá-Vicente *et al*., 2008a, 2012; Sieber and Grünig, 2013; Keim *et al*., 2014), which are often co-dominated by species related to *Fusarium*, Pleosporaceae and *Cadophora*. The distinctive traits of these lineages are suggestive of different niche occupancies, therefore it is possible to associate the phylogenetic diversity of endophytic communities with processes of competition or complementarity among species. The co-occurrence of endophytes not sharing functional characteristics is indicative that competition is a main driver of community assembly, because functional complementarity reduces competition and promotes co-existence (Maherali and Klironomos, 2007, 2012). Conversely, communities shaped by environmental filtering usually show phylogenetic clustering of species with similar traits selected by the limiting factors. This could explain the low diversity in root-endophytic communities subject to salt stress, where pleosporaceous endophytes become enriched while otherwise dominant fusaria are absent (Maciá-Vicente *et al*., 2008a, 2012). Although functional complementarity among species enhances ecosystem function, the trait similarity of phylogenetically related endophytes (for example, different *Fusarium* spp. co-occurring in the same root) can lead to functional redundancy. This has shown to provide stability to plant–endophyte symbioses, because it prevents the loss of symbiotic functions with the replacement of

fungal species across environmental gradients (Maherali and Klironomos, 2007).

Conclusions

Non-mycorrhizal fungal endophytes are pervasive in roots, hence they are likely to affect plant abundance and productivity in natural communities. Although our experimental system was artificial, it was adequate to address our objective to test interactions between plants and a large number of endophytic species. Under these conditions most endophytes behaved as weak parasites, but their performance varied across plant species and fungal taxa. Diverging endophyte lineages have evolved distinct strategies of plant symbiosis, but their associations were often variable, suggesting that they are subject to local processes of selection. Part of the variation in the interactions was explained by convergent fungal traits that differentiate categories of endophytes with potentially distinct niches. The functional complementarity of strains belonging to different groups is predicted by the structure of natural root-endophytic communities. The characterization of the endophytic diversity into potential functional groups will aid in the testing of further questions about their role in ecosystems. In particular, the assessment of the responses of plant–endophyte interactions to (a)biotic factors, including combinations of endophytes with different degrees of trait similarity and shared evolutionary history, will help unravel context-dependent symbioses adaptive under natural conditions.

Conflict of Interest

The authors declare no conflict of interest.

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(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende KG: 15% isolation of fungal strains, molecular characterization of fungal strains, preperation of strains for metabolic analysis
Co-Author ZCA: 50% preparation of crude extracts, HPLC-MS/MS and NMR analyses of fungal secondary metabolites
Co-Author JGMV: 25% isolation of fungal strains, molecular and morphological characterization of fungal strains, preparation of strains for metabolic analysis, phylogenetic analyses
Co-Author MK: 10% toxicity assays of purified compounds

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende KG: 10% collection of plant samples for fungal isolation, collection of sequencing data
Co-Author ZCA: 50% collection of data from chemical profiles, preparation of figures
Co-Author JGMV: 35% % collection of plant samples for fungal isolation, comparison and collection of sequence data from public databases, preparation of voucher strains
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(4) zur Analyse und Interpretation der Daten

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Diversity of exophillic acid derivatives in strains of an endophytic *Exophiala* sp.

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ABSTRACT

Members of the fungal genus *Exophiala* are common saprobes in soil and water environments, opportunistic pathogens of animals, or endophytes in plant roots. Their ecological versatility could imply a capacity to produce diverse secondary metabolites, but only a few studies have aimed at characterizing their chemical profiles. Here, we assessed the secondary metabolites produced by five *Exophiala* sp. strains of a particular phylotype, isolated from roots of *Microthlaspi perfoliatum* growing in different European localities. Exophillic acid and two previously undescribed compounds were isolated from these strains, and their structures were elucidated by spectroscopic methods using MS, 1D and 2D NMR. Bioassays revealed a weak activity of these compounds against disease-causing protozoa and mammalian cells. In addition, 18 related structures were identified by UPLC/MS based on comparisons with the isolated structures. Three *Exophiala* strains produced derivatives containing a β-D-glucopyranoside moiety, and their colony morphology was distinct from the other two strains, which produced derivatives lacking β-D-glucopyranoside. Whether the chemical/morphological strain types represent variants of the same genotype or independent genetic populations within *Exophiala* remains to be evaluated.

1. Introduction

Fungi are among the best producers of low molecular weight molecules with a great variety of bioactivities. This is likely due to the complexity of their habitats where they interact with a broad diversity of microorganisms through processes such as antibiosis, quorum sensing or elicitation (Brakhage and Schroeckh, 2011; Hogan, 2006). Endophytic fungi inhabit the interior of plant tissues without causing apparent harm to their hosts, and have been long studied as a particular group for drug discovery because they possess a number of desirable features (Higginbotham et al., 2013; Kusari et al., 2012; Peláez et al., 1998; Schulz et al., 2002). Firstly, they possess a huge and as yet largely untapped taxonomic and functional diversity, and their specific preferences across plant hosts and target tissues determine a partitioned distribution likely to increase their chemical diversity. Secondly, they establish an interaction with their host plants, which requires a metabolic toolbox and a constant production of compounds and effectors for the maintenance of the symbiosis. Thirdly, many endophytes are easy to isolate and culture in fermentation processes thus facilitating the exploitation of their products in industrial set-ups.

In this work we investigated the chemical constituents of endophytic strains within the genus *Exophiala* (Herpotrichiellaceae, Chaetothyriales) isolated from roots of the brassicaceous plant *Microthlaspi perfoliatum* (L.) F.K. Meyer, which despite of having a wide distribution range has only been recently studied with respect to its endophytes (Keim et al., 2014). The strains were analyzed in the context of an ongoing screening for novel fungal secondary metabolites, from a collection of fungal endophytes that originate from diverse plant species, geographical locations and ecological contexts. The genus *Exophiala* contains polymorphic

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and dark-pigmented species, many of them capable of producing budding cells (these are commonly known as black yeasts). They are cosmopolitan and occur frequently in plant material, soil and water environments, but also as opportunistic pathogens of animals (de Hoog et al., 2011; Haase et al., 1999). Like other melanized fungi, they are often associated with extreme environments, and are frequently described as dark-septate endophytes colonizing roots of various plants, although they are generally not considered to constitute a proportionally important component in most endophytic communities (Addy et al., 2005; Sterflinger, 2006). Some root-endophytic strains of *Exophiala* have shown a capacity to confer benefits to their host plants by increasing their tolerance to abiotic stresses (Khan et al., 2011; Li et al., 2011).

Only a few studies have been carried out to identify natural products produced by *Exophiala*. Of the 42 species listed in Index Fungorum (http://www.indexfungorum.org), reports on secondary metabolites exist only for a few of them. These studies have revealed a variety of metabolites, a selection of which (compounds **1**–**18**) is shown in Fig. 1. A resorcinol compound, most likely of polyketide origin (1-(3,5-dihydroxyphenyl)-4-hydroxypentan-2-one; **1**), a chromone derivative (7-methoxy-2,3,6-trimethylchromone; **2**), indole alkaloids (brevianamide F and N_b-acetyltryptamine; **3**,**4**), and exophillic acid (**5**) have been isolated from *Exophiala pisciphila* (Ondeyka et al., 2003; Wang et al., 2011). Exophialin (**6**), 8-hydroxyexophialin (**7**) and pityriacitrin (**8**) have been isolated from *E. dermatitidis* (Kindler et al., 2010). Seven compounds (**9**–**15**) from *E. oligosperma* have been identified as 2-phenoxynaphthalene (**9**), (2*S*,3*R*,4*E*,8*E*)-1-O-β-D-glucopyranosyl-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl]amino-9-methyl-4,8-octadeca-diene (**10**), four steroids ((22*E*,24*R*)-ergosta-7,22-dien-3β,5α,6β-triol, (22*E*,24*R*)-3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one, (22*E*,24*R*)-5α,6α-epoxy-ergosta-8,22-dien-3β,7α-diol and (22*E*,24*R*)-ergosta-4,6,8(14),22-tetraen-3-one; **11**–**14**), and euphorbol (**15**) (Li et al., 2011). Chlorohydroaspyrones A (**16**) and B (**17**) were isolated from a marine-derived *Exophiala* sp. (Zhang et al., 2008). Exophilin A (**18**), an unusual hydroxy fatty acid, has been isolated from *E. pisciphila*. Mono- and polyunsaturated fatty acids have been also reported in members of the genus.

We aimed to identify novel chemical structures from different fungal strains within *Exophiala*. For this purpose, we selected five isolates obtained as endophytes from roots of *M. perfoliatum* collected at different areas across Europe, but which belonged to the same phylotype according to partial ribosomal DNA sequences. Using this approach we were able to explore the variability of the secondary metabolite production profiles across strains and/or ecological origins.

2. Results

2.1. Characterization of the strains

The five strains studied could be classified within the genus *Exophiala* by both morphological and molecular methods. All of them showed traits typical of this fungal group, including a slow-growing, dark-gray mycelium on PDA, dome-shaped and hairy or slimy and flat colonies, and torulose hyphae. Conidia were scarce in all strains, and when present they were ellipsoidal to short cylindrical, and occasionally septate. They lacked yeast cells and did not grow at 37 °C, which is characteristic of species like *Exophiala equina*, *E. pisciphila* and *Exophiala salmonis* (de Hoog et al., 2011, 1995).

The ribosomal ITS region is regarded as an informative marker for species delimitation within *Exophiala*, and the phylogeny we calculated is in accordance to others previously established for this genus (de Hoog et al., 2011; Zeng and de Hoog, 2008). The phylogenetic analysis clustered all five strains in a strongly supported clade (bootstrap values of 99% and 93% for the ML and NJ trees, respectively) alongside other sequences from diverse origins, but separately from all sequences from type strains for *Exophiala* or other related taxa (Fig. 2, see also S1). The closest identified species to our strains were *E. equina* (strain CBS 119.23; de Hoog et al., 2011) and *E. salmonis* (CBS 157.67; Carmichael, 1966), with a strongly supported separation in the ML phylogeny (Fig. 2). The following branch of the tree included the species *E. pisciphila* (CBS 537.73; McGinnis and Ajello, 1974), although this was not supported by the bootstrap analyses. The clear separation of our strains from *E. equina* and *E. salmonis* precluded the assignment to any species, and thus a generic classification was preferred.

We consider that the five strains belong to a same species, according to the high similarity of their ITS sequences. Although both strains from Bulgaria (P1910 and P1860) formed a clade slightly apart from the remaining strains, this was not supported by a substantial difference in their sequences (Fig. 2). In spite of their close molecular affinities, the strains displayed divergent morphological features (Fig. 3). Strains P1095 from France and P2854 from Germany displayed a dry thallus with abundant aerial mycelium. On the other hand, strains P1860 and P1910 from Bulgaria, and P2772 from Germany had their colonies covered by a slimy material and lacked aerial mycelium. The slimy phenotype was accompanied by a profuse presence of extracellular droplet-like structures that were glossy under phase contrast microscopy, and which were nearly absent in the other morphotype (Fig. 3). Similar droplets were present forming intra-hyphal chains in both morphological groups. These morphologies were consistent after repeated recoveries of the strains from the original isolation plates or from stock cultures, and hence this trait is most probably not due to differences in the handling of the cultures.

2.2. Isolation and identification of Exophiala sp. secondary metabolites

The ethyl acetate extract of strain P1860 was chromatographed by semi-preparative RP-HPLC to furnish exophillic acid (**5**, 2 mg/plate) and its corresponding monomeric form (**19**, 0.2 mg/plate). Compound **20** (1 mg/plate) was isolated from the ethyl acetate extract of strain P1095 by RP-HPLC. Compound **19** was obtained as a slightly violet oil, and its ESIMS showed a molecular ion $[M-H]^-$ at m/z 441.2136, in accordance with the molecular formula $C_{22}H_{33}O_9$. The IR spectrum showed the presence of a carboxyl (1735 cm^{-1}) and aromatic (1655 cm^{-1}) moieties. ^1H NMR analysis showed two doublets corresponding to aromatic meta-coupled protons (δ_H 6.38, 6.56, J = 5.0 Hz) and a triplet (δ_H 0.9, J = 10.0 Hz) corresponding to a terminal methyl group. The ^{13}C NMR spectrum obtained in deuterated methanol (Table 1) exhibited resonances for 22 carbons, including an ester carbonyl at δ 172.6 (C-1′), a methyl at δ_C 14.4 CH_3 (C-16′) and eight methylene groups between δ_C 23.7 and δ_C 34.9, characteristic of methylene groups present in a long alkyl chain. The aromatic ring was characterized by signals corresponding to quaternary carbons at δ_C 118.2, 160.6, 157.5, and 144.3 (C-2′, C-3′, C-5′, and C-7′ respectively) and two CH groups at 102.2 (C-4′), 111.7 (C-6′) showing correlations with δ_H 6.56 and δ_H 6.38 ppm respectively in HSQC. The value of the coupling constant between the hexose protons (J = 7.5 Hz; Table 1) and the chemical shifts of C-1″ to C-6″ (δ_C 103.6, 75.0, 77.7, 71.2, 78.3, and 62.5, respectively) were in good agreement with reported data for a β-D-glucopyranosyl moieties (Fig. 4; Ondeyka et al., 2003; Waki et al., 2007).

Interpretation of ^1H, ^{13}C, and 2D NMR (COSY–HSQC, and HMBC; Figs. S2–S6) spectra allowed the structure elucidation of compound **19** to be 4-hydroxy-2-nonyl-6-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzoic acid.

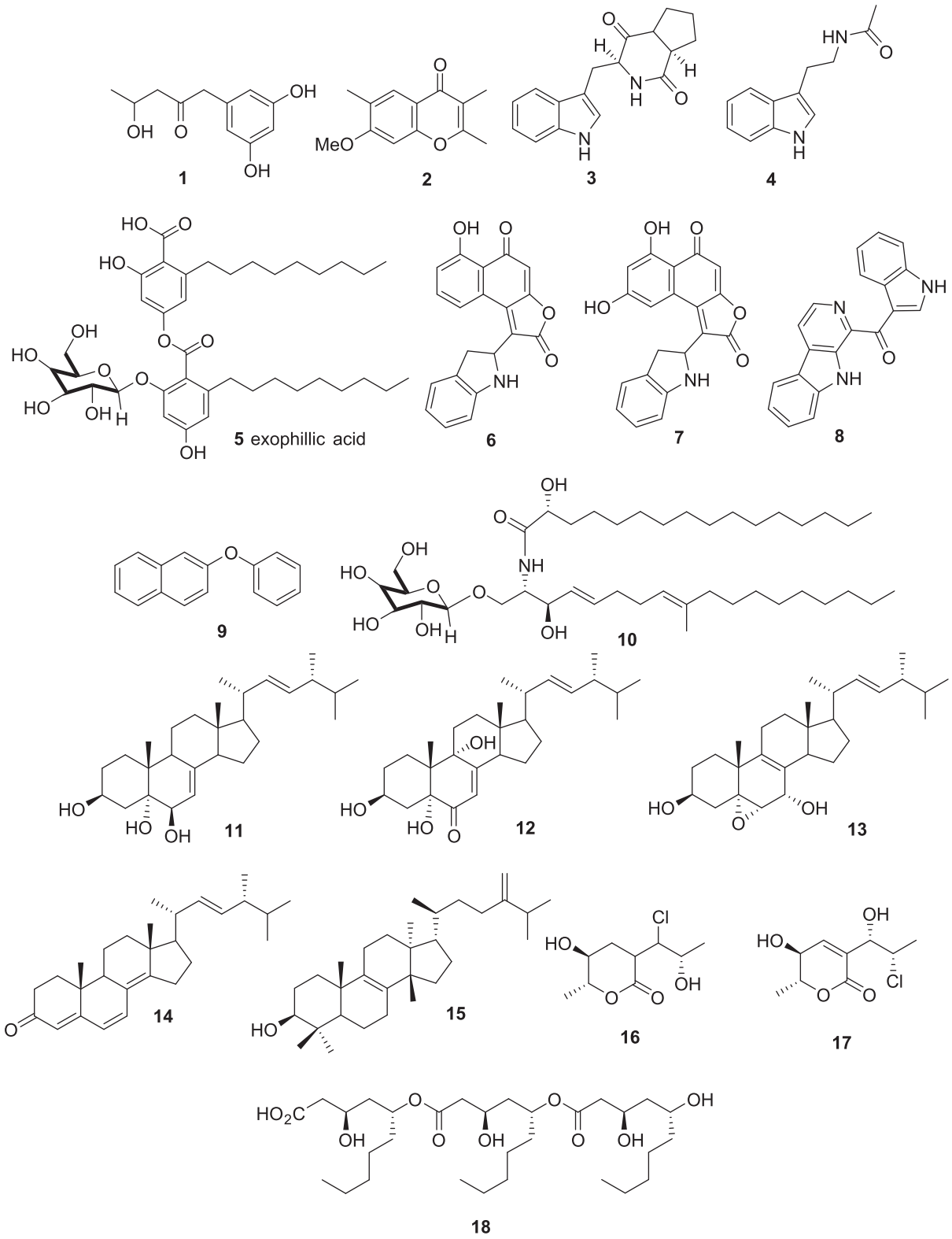


Fig. 1. Metabolites previously identified in *Exophiala* species. **1**–**5**, compounds isolated from *E. pisciphila* by Ondeyka et al. (2003), Wang et al. (2011): a resorcinol compound probably of polyketide origin (1-(3,5-dihydroxyphenyl)-4-hydroxypentan-2-one; **1**), a chromone derivative (7-methoxy-2,3,6-trimethylchromone; **2**), indole alkaloids (brevianamide F and N_b-acetyltryptamine; **3**,**4**), and exophillic acid (**5**). **6**–**8**, compounds isolated from *E. dermatitidis* by Kindler et al. (2010): exophialin (**6**), 8-hydroxyexophialin (**7**) and pityriacitrin (**8**). **9**–**15**, compounds from *E. oligosperma* isolated by Li et al. (2011): 2-phenoxynaphthalene (**9**), (2*S*,3*R*,4*E*,8*E*)-1-O-β-D-glucopyranosyl-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl]amino-9-methyl-4,8-octadeca-diene (**10**), four ergosterol derivatives ((22*E*,24*R*)-ergosta-7,22-dien-3β,5α,6β-triol, (22*E*,24*R*)-3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one, (22*E*,24*R*)-5α,6α-epoxy-ergosta-8,22-dien-3β,7α-diol and (22*E*,24*R*)-ergosta-4,6,8(14),22-tetraen-3-one; **11**–**14**), and euphorbol (**15**). **16**–**17**, chlorohydroaspyrones A (**16**) and B (**17**) isolated from an *Exophiala* sp. of marine origin by Zhang et al. (2008). **18**, hydroxyl fatty acid exophilin A from *E. pisciphila* by Doshida et al. (1996).

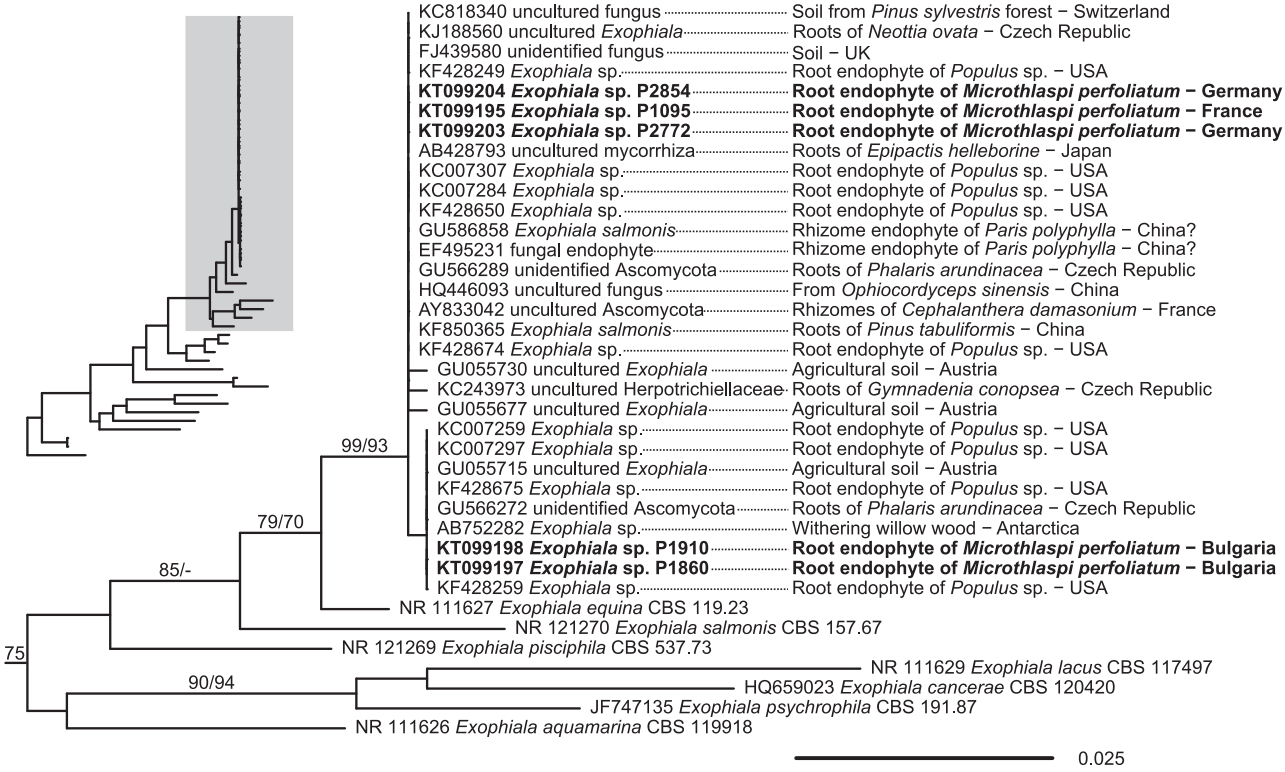


Fig. 2. Maximum Likelihood ITS rDNA phylogeny of the endophytic *Exophiala* sp. strains in this study (in bold), in relation to their 25 BLAST closest matches (including substrate and country of origin; '?' indicates uncertainty in the country of origin) and the 25 closest reference strains at NCBI GenBank (GenBank accession numbers before sequence descriptions; voucher numbers of reference strains after species names). Only a portion of the whole phylogenetic tree (gray rectangle in the inset) is shown to better depict the relationship of the strains with their closest sequences. For a full version of the ML tree see Fig. S1. Bootstrap support values >70% for the maximum likelihood and for a Neighbor-Joining analysis (ML/NJ) are shown next to each tree branch.

The corresponding dimeric form, exophillic acid (**5**), has been previously isolated (Ondeyka et al., 2003). Interestingly, we isolated a novel dimeric structure (**20**) from another *Exophiala* sp. strain, P1095. Compound **20** displayed in ESIMS a molecular ion $[M-H]^-$ at *m/z* 541.3195, consistent with the molecular formula $C_{32}H_{45}O_7$. Thus the molecular weight of compound **20** was 162 Da less than exophillic acid, a shift that is in accordance with the loss of the β-D-glucopyranosyl moiety (Fig. 5). Subsequently, the structure of compound **20** was established by 1D and 2D NMR (Figs. S7–S11) as 4-((2,4-dihydroxy-6-nonylbenzoyl)oxy)-2-hydroxy-6-nonylbenzoic acid.

A variety of related structures were also identified by analytical HPLC/MS based on comparisons with the above described authentic compounds (**5**, **19**, and **20**). All additional secondary metabolites identified in the *Exophiala* strains are shown in Tables 2 and 3 (for a detailed description see Tables S14 and S15). All of the newly identified metabolites (**21**–**29**) possessing a β-D-glucopyranoside are present in strains with a slimy phenotype, namely P1860, P1910 and P2772 (Table 2). Conversely, metabolites **30**–**38** identified in strains that present a dry phenotype with aerial mycelium, P1095 and P2854, lacked the β-D-glucopyranoside residue (Table 3). In general derivatives with double bonds and hydroxyl groups in the alkyl side chains have been identified according to MS2 fragmentation data (Tables 2 and 3). To confirm the presence of double bonds in the identified structures, crude extracts from P1860 and P1095 were derivatized using dimethyl disulfide (DMDS) in a iodine solution (Francis and Veland, 1987), resulting in the detection of the corresponding dimethyl disulfide adduct in HPLC–MS analysis (identified by a mass increase of 94 Da; Fig. S12).

The positions of these double bonds were determined as follows: crude extracts were methanolyzed under acidic conditions followed by addition of DMDS as described previously (Bode et al., 2006; Francis and Veland, 1987), and then were subjected to GC/MS analysis. Due to the low amount of unsaturated derivatives (Tables 2 and 3) we were only able to determine the potential position of a double bond in compounds **22**, **31**, and **32**. The specific fragments allowed the localization of the double bond between C-9 and C-10 (Fig. S13).

The position of the hydroxyl groups have not been determined as they are present only in trace amounts.

Compound 19: slightly violet oil; UV (MeOH) λ_{max} 220, 290 nm; IR (film, MeOH) ν_{max} 3410, 2945, 1735, 1655, 1450, 1260, 1205, 1045, 820 cm^{-1}; HRESIMS *m/z* 441.2136 (*calcd* for $C_{22}H_{34}O_9 - H^-$, 441.2130, Δ = 1.36 ppm); t_R (HPLC) 8.6 min.

Compound 20: yellow oil; UV (MeOH) λ_{max} 215, 280, 310 nm; IR (film, MeOH) ν_{max} 3265, 1670, 1235, 1150, 860, 755; HRESIMS 541.3169 *m/z* (*calcd* for $C_{32}H_{45}O_7 + H^-$, 541.3171, Δ = 0.37 ppm); t_R (HPLC) 14.1 min.

2.3. Bioactivity of Exophiala sp. secondary metabolites

The major compounds **5**, **19** and **20** were tested against the causative agents of tropical neglected diseases *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*, and their cytotoxicity was tested against mammalian L6 cells. For *T. brucei*, **19** had the best activity with an IC_{50} of 16 μg ml^{-1}, whereas the dimeric compounds **5** and **20** showed activities above 35 μg ml^{-1}. A similar activity of all compounds was observed against *P. falciparum* with 25, 22 and 26 μg ml^{-1} for **5**, **20**, and **19**, but they only had a weak bioactivity against *T. cruzi* and L6 cells (30–60 μg ml^{-1}). A clear difference between the glycosylated and non-glycosylated compounds was observed against *L. donovani*. Whereas **5** and **19** showed an IC_{50}

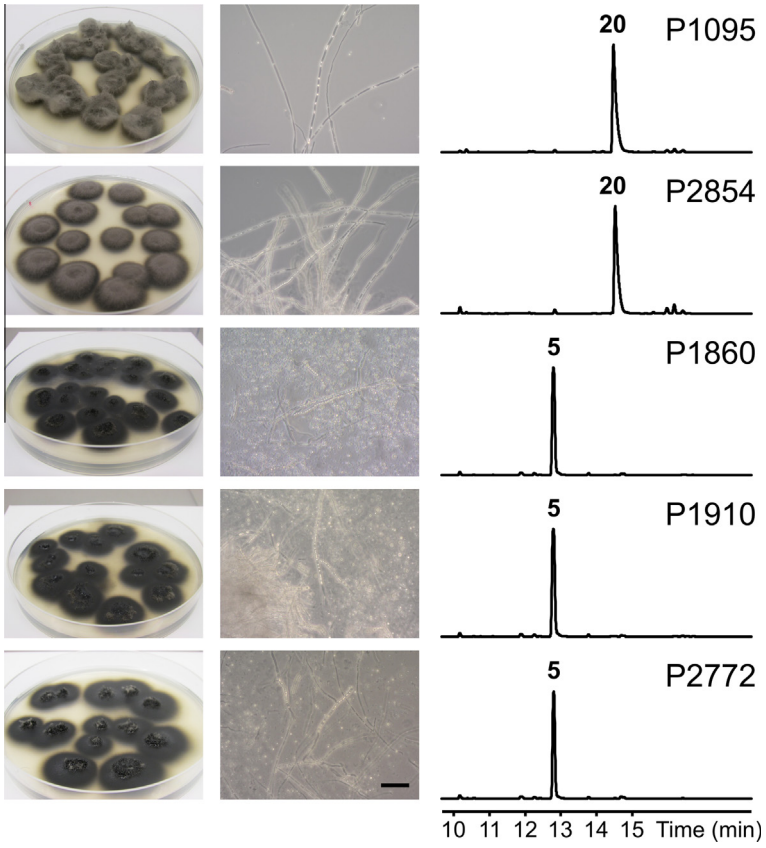


Fig. 3. Morphological and chemical features of the endophytic *Exophiala* sp. strains included in this study. Left, pictures of 30-day-old colonies of each strain on potato dextrose agar, showing the differential dry or slimy morphological types described in the text. Middle, phase-contrast microscopy images of the mycelium of each strain, showing presence of intra-hyphal droplets in all cases, and abundant extra-cellular droplet production in the slimy morphotype. Bar at bottom represents 20 μm. Right, base peak UV chromatograms at λ_{max} = 280 nm for each strain displaying predominant compounds **20** and **5**.

of 55 and 63 μg ml^{-1}, **20** showed an IC_{50} of 16 μg ml^{-1}. However, compared to standard drugs against these parasites, the activity of all tested compounds was at least 100-fold lower.

3. Discussion

We have identified 21 secondary metabolites from five strains of a root-endophytic *Exophiala* sp. All are derivatives of exophillic acid (compound **5**), a dimeric 2,4-dihydroxy alkyl benzoic acid first reported from one of the closest relatives to our strains, *E. pisciphila* (Ondeyka et al., 2003). A large part of the structural diversity within this suite of identified compounds is due to the presence or absence of a β-D-glucopyranoside residue at position C-3′ of the aromatic group. The observed diversity among these derivatives can be explained as intermediate steps in the biosynthetic pathway leading to the main compounds. For example, monomeric structures **19** and **37** could lead to the predominant structures **5** and **20** upon dimerization. Compounds **36** and **38** could represent potential intermediates of non-predominant structures **31** and **33**, respectively. Other compounds with a hydroxyl-alkyl chain like **24** could result from hydration of unsaturated alkyl chain, as in **22** or vice versa, a process likely to occur during the biosynthesis (Cheikh-Ali et al., 2011). Interestingly, unsaturated and hydroxylated fatty acids have been previously described in *Exophiala* spp. (Doshida et al., 1996; Hsueh et al., 2001; Rene et al., 2012).

The first and—to our knowledge—only study on exophillic acid was related to its inhibitory activity against HIV-1 integrase, an enzyme critical in the replication and spread of the human immunodeficiency virus (Ondeyka et al., 2003). No other biological activities are known for this compound and hence its potential physiological or ecological role remains to be evaluated. Structurally similar metabolites have been reported from fungi unrelated to *Exophiala*, such as aquastatin A in *Fusarium aquaeductum* and *Sporothrix* sp., KS-501 and KS-502 in *Sporothrix* sp., and TPI 1 in *Hypomyces* sp. and *Nodulisporium* sp. (Kwon et al., 2009; Nakanishi et al., 1989; Ondeyka et al., 2003; Yasuzawa et al., 1990). Again, these have therapeutically relevant bioactivities but unknown roles in the fungi that produce them. Among these compounds, aquastatin A has shown an antibiotic activity towards multiple bacterial groups via inhibition of fatty acids biosynthesis (Hamano et al., 1993; Kwon et al., 2009). Like exophillic acid, aquastatin A possesses a monosaccharide moiety in its structure, in this case a β-galactose residue, whose removal does not affect its antimicrobial activity (Kwon et al., 2009).

Two of the derivatives we have identified in our *Exophiala* sp. strains (compounds **37** and **38**) have been previously detected in *Gibberella zeae* and *Neurospora crassa*, and are instances of polyketide resorcylic acids that are widespread and very diverse in fungi (Funa et al., 2007; Zhou et al., 2008). Compound **36** has also a similar structure but with a longer fatty acid side chain, representing the longest chain of this sort found in natural resorcylic acid derivatives (Wang et al., 2013). Fungal polyketides of this kind often have potent antibiotic activities against competitor microorganisms in natural substrata, although there are also examples of their participation in synergistic interactions with bacteria or algae (Schroeckh et al., 2009; Zhou et al., 2011). Other compounds isolated previously from *Exophiala* species have shown antimicrobial

Table 1
NMR spectroscopic data (500 MHz, CD_3OD) of compounds **19** and **20**.

Position	Compound **19**		Compound **20**	
	δ_C	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1			170.5 qC	
2			113.5 qC	
3			154.8 qC	
4			112.2 CH	6.29, d (5.0)
5			149.4 qC	
6			116.1 CH	6.57, d (5.0)
7			149.0 qC	
8			37.9 CH_2	2.89, m
				2.96, m
9			33.6 CH_2	1.63, m
10			31.0 CH_2	1.32, m
11			30.8 CH_2	1.32, m
12			30.7 CH_2	1.32, m
13			30.7 CH_2	1.32, m
14			33.0 CH_2	1.32, m
15			23.7 CH_2	1.32, m
16			14.5 CH_3	0.86, t (10.0)
1′ (C=O)	172.6 qC		166.0 qC	
2′	118.2 qC		105.3 qC	
3′	160.6 qC		164.2 qC	
4′	102.2 CH	6.56, d (5.0)	102.0 CH	6.23, d (5.0)
5′	157.5 qC		164.4 qC	
6′	111.7 CH	6.38, d (5.0)	109.1 CH	6.63, d (5.0)
7′	144.3 qC		149.0 qC	
8′	34.9 CH_2	2.55, m	36.8 CH_2	2.89, m
		2.68, m		2.96, m
9′	33.1 CH_2	1.58, m	33.0 CH_2	1.63, m
10′	30.7 CH_2	1.31, m	30.6 CH_2	1.32, m
11′	30.6 CH_2	1.31, m	30.6 CH_2	1.32, m
12′	30.5 CH_2	1.31, m	30.5 CH_2	1.32, m
13′	30.4 CH_2	1.31, m	30.5 CH_2	1.32, m
14′	32.5 CH_2	1.31, m	33.1 CH_2	1.32, m
15′	23.7 CH_2	1.31, m	23.8 CH_2	1.32, m
16′	14.4 CH_3	0.9, t (10.0)	14.5 CH_3	0.89, t (10.0)
1″	103.6 CH	4.85, d (7.5)		
2″	75.0 CH	3.47, t (7.5)		
3″	77.7 CH	3.44, m		
4″	71.2 CH	3.40, m		
5″	78.3 CH	3.42, ddd (2.0, 5.5, 7.5)		
6″	62.5 CH_2	3.72, d/d (5.0, 10.0)		
		3.90, d/d (1.5, 10.0)		

activities (Doshida et al., 1996; Zhang et al., 2008). In our study, no strong activities were detected against animal-parasitic protozoa or mammalian cells, but further tests on microorganisms frequent in the strains' habitat of origin would be necessary to assess their potential role in microbial communication.

Metabolites described in different *Exophiala* species (compounds **1**–**4**, and **6**–**18**; Doshida et al., 1996; Kindler et al., 2010; Li et al., 2011; Wang et al., 2011; Zhang et al., 2008) were not detected in our extracts. While it is possible that differences in the production of these metabolites are owing to characteristics of the particular species, we cannot discount a potentially considerable influence of the growth conditions prior to extraction and analysis. The above compounds were reported from strains grown in liquid media, or on solid media with specific nutritional conditions to fulfill experimental needs. In our case, we used a common solid medium for fungal cultivation (PDA) with fixed incubation conditions, since this study is part of an ongoing screening involving hundreds of isolates that requires a standardized cultivation protocol, able to sustain growth of a multitude of fungal taxa. As previously noted, changes in incubation conditions have profound effects on the regulation of gene clusters implicated in the synthesis of secondary metabolites, and hence the analysis of a single strain under multiple cultivation conditions would likely increase the diversity of compounds that can be observed (Bode et al., 2006; Fox and Howlett, 2008; Paranagama et al., 2007).

Our classification places the endophytic strains in this work within the *salmonis* clade of *Exophiala* defined by de Hoog et al. (2011). This clade includes *E. pisciphila*—in which exophillic acid was first identified—together with other mesophilic or psychrotolerant species commonly found in freshwater environments, and occasionally parasitizing cold-blooded animals. Interestingly, most of the closest BLAST hits to our strains came from root or rhizome-associated fungi, often isolated as endophytes (Bukovská et al., 2010; Julou et al., 2005; Ogura-Tsujita and Yukawa, 2008; Těšitelová et al., 2013). This could imply that our strains belong to a clade with a habitat preference towards soil and rhizospheric environments, with the capacity to penetrate and dwell within roots of a variety of host plants. Several *Exophiala* species occur in soils or other nutrient-poor substrata (Bates et al., 2006; de Hoog et al., 2011), which can constitute a reservoir of inoculum for a later colonization of roots. Evaluating the presence of similar metabolites across *Exophiala* species and adjacent taxa would be very valuable to infer a broader ecological meaning, by collating chemical and genetic differences with habitat preferences of these fungi. Moreover, because exophillic acid has only been identified in the related *E. pisciphila* but not in other distant members of *Exophiala*, evaluating the production of similar compounds by sister species to our strains could help to determine their validity as chemotaxonomic markers for this clade, as has been shown for multiple other compounds and fungal taxa (e.g., Frisvad et al., 2008; Stadler et al., 2003; Stadler and Hellwig, 2005).

The secondary metabolite profiles of different *Exophiala* strains were dominated by either of the exophillic acid derivatives with or without the β-D-glucopyranoside residue, a feature that was linked to morphological differences of their colonies. Strains producing predominantly derivatives with the β-D-glucopyranoside group displayed slimy colonies with scarce aerial mycelium, and produced abundant extracellular droplet-like bodies. In contrast, strains producing metabolites that lack the monosaccharide moiety had dry colonies with abundant aerial mycelium, and showed a far restricted production of extracellular droplets. No diglycosylated compounds were detected, but the presence of compounds

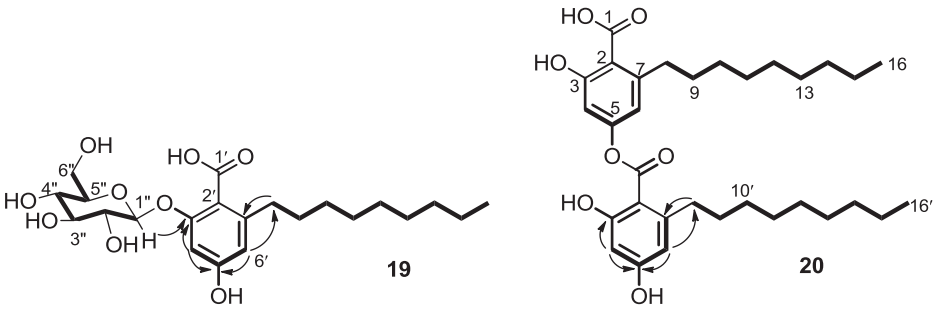


Fig. 4. Selected ^1H–^1H COSY (bold lines) and HMBC (arrows) correlations of compounds **19** and **20**.

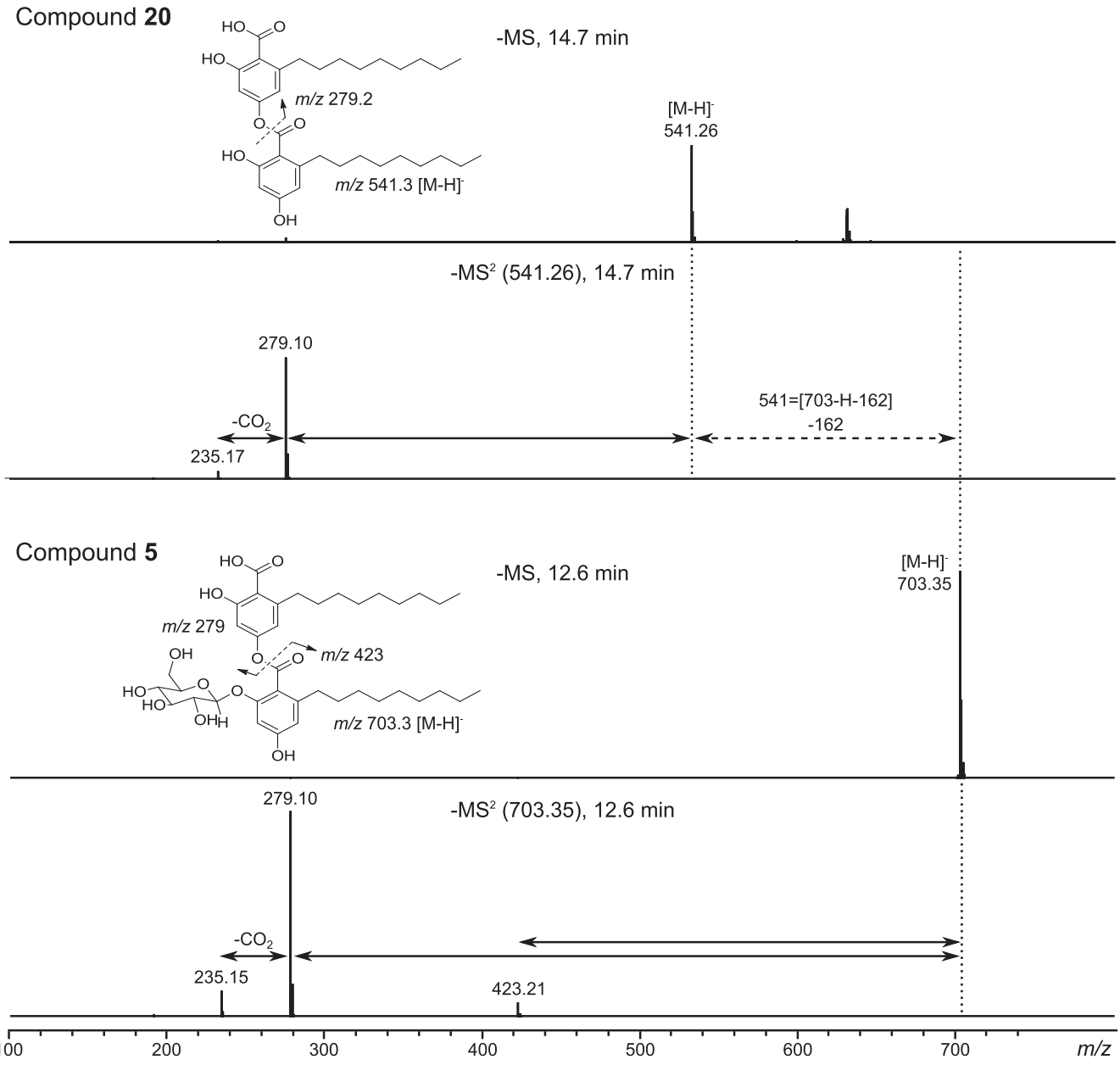


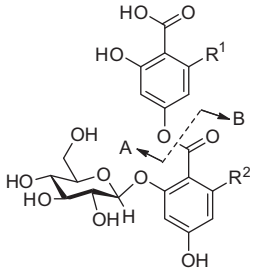
Fig. 5. Mass spectrometry spectra and major fragments of **5** and **20**. For each compound, the upper and lower plots show the MS and MS2 spectra, respectively. Continuous arrows represent the fragmentation of each compound, and the dashed arrow represents the mass difference between compounds **20** and **5**.

like **16** might indicate that the glycosylation takes place prior to dimerization and is specific for monoglycosylation. A definitive conclusion on whether glycosylation is associated to distinct genotypes or to a context-dependent expression cannot be drawn from our results. Strain P1095 had dry colonies and was the only *Exophiala* isolate obtained from a site in France. Two of the strains with a slimy morphology originated from the same site in Bulgaria (P1910 and P1860), from roots of two individual *M. perfoliatum* plants growing a few meters apart in a same soil type. It is likely that they represent clonal colonies or ramets of a same fungal genotype, as root endophytes can occur both in plant roots and the surrounding soil, and colonize adjacent plants (Maciá-Vicente et al., 2012). The two strains isolated from Germany (P2772 and P2854) originated from plant populations separated by about 50 km. In spite of their relative proximity they presented contrasting morphologies and chemical profiles. It is feasible that they represent genetically different populations within the same species, as each chemical/morphological type was obtained from an isolated population of the host plant. On the other hand, it is also possible that the strains are morphological variants of a unique genotype, since previous works have shown that populations of root endophytes might extend across large geographical areas (Queloz et al., 2011). Besides, members of the Herpotrichiellaceae are pleomorphic and often display diverse morphological features within a single lineage, what makes recognition rather difficult by morphological means (Untereiner and Naveau, 1999).

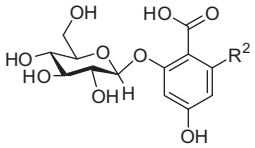
4. Concluding remarks

Our results show that exophillic acid and related derivatives are among the main secondary metabolites produced by the

Table 2
Natural derivatives identified from *Exophiala* sp. strain P1860, and their amount relative to a maximum production of **5** (100%).



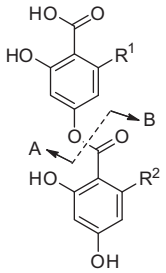
5,21-29



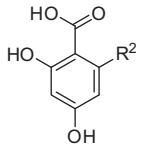
19,27

No	Amount (%)	R^1	*A* (*m*/*z*)	R^2	*B* (*m*/*z*)
5	100		279		423
21	11		279		396
22	5		279		421
23	0.2		295		439
24	5		279		439
25	3		279		437
26	3		279		394
28	1		279		368
29	0.1		279		524
19	4				
27	0.1				

Table 3
Natural derivatives identified from *Exophiala* sp. strain P1095, and their amount relative to a maximum production of **20** (100%).



20,30-35



36-38

No	Amount (%)	R^1	*A* (*m*/*z*)	R^2	*B* (*m*/*z*)
20	100		279		279
30	2		279		379
31	3		277		379
32	8		279		277
33	3		279		251
34	6		279		295
35	0.4		295		295
36	2				
37	12				
38	0.2				

root-endophytic *Exophiala* sp. herein studied. Strains clustered into two groups based on their morphology and secondary metabolites profiles. Based on the current sampling, unequivocal conclusions about the relationship among secondary metabolite profiles, fungal morphology and ecological origin of the strains cannot be drawn. However, our work shows that geographically distant strains from a same fungal group produce similar compounds, but present slight differences in their structure that might reflect either genetic or phenotypic adaptations.

5. Experimental

5.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 500 spectrometer using deuterated methanol as solvent. Infrared (IR) spectra were recorded on a PerkinElmer Spectrum 100 FT-IR Spectrometer.

The identification of new derivatives was performed by ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–ESIMS) in a Ultimate 3000 system (Dionex, Idstein, Germany) by using a RP C_{18} BEH Acquity UPLC column (50 mm × 2.1 mm × 1.7 μm, Waters, USA), coupled with an AmazonX ion trap MS (Bruker, Bremen, Germany). Precise mass measurements were carried out by HR-ESI-MS using a linear gradient from 5% to 95% ACN (0.1% formic acid) over 12 min with a flow rate of 0.4 ml min^{-1}. Masses were detected with a Bruker micrOTOF.

Compounds **5**, **19** and **20** were isolated with a semi-preparative RP-HPLC equipped with a Waters (Milford, MA, USA) XBridge™ Semiprep C18 column (5 μm OBDTM 19 × 150 mm, S/N) and a Waters HPLC–MS-system coupled with Waters 3100 Mass Detector, and Waters 2998 Photodiode Array Detector, using a linear gradient from 5% to 95% ACN (0.1% formic acid) over 32 min with a flow rate of 26 ml min^{-1}.

Gas chromatography was performed using an Agilent 780A/597C/GC/MS system equipped with a DB5ht column (length of 30 m, 0.25 mm of an inner diameter, 0.1 μm in strength of stationary phase film; Agilent, Waldbronn, Germany). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. Ionization energy was 70 eV. One microliter of each sample was injected into the gas chromatograph in splitless mode with 250 °C inlet temperature. Initial oven temperature was 70 °C for 5 min followed by 5 °C min^{-1} to 300 °C, and 5 min at 300 °C.

5.2. Fungal material

Fungal strains P1095, P1860, P1910, P2772 and P2854 were isolated as endophytes from roots of the annual plant *M. perfoliatum* during a sampling campaign in spring 2013 (Glynou et al., unpublished). Strain P1095 was isolated from a population of *M. perfoliatum* in eastern France (47.41 N, 6.56 E); P1860 and P1910 were obtained from different plant specimens of the same plant population in Bulgaria (42.70 N, 22.83 E); and P2772 and P2854 originated from two *M. perfoliatum* specimens in plant populations growing ca. 50 km apart in Germany (49.68 N, 10.00 E and 49.54 N, 9.34 E, respectively). We identified all strains as *Exophiala* sp. by assessing micro- and macro-morphological characters, and by sequencing their internal transcribed spacers (ITS) of the ribosomal DNA (GenBank accession numbers KT099195, KT099197, KT099198, KT099203 and KT099204) and comparing them with reference sequences. The strains have been deposited in the reference collection of CBS-KNAW Fungal Biodiversity Centre under accession numbers CBS 140398, CBS 140399, CBS 140400, CBS 140401 and CBS 140402.

5.3. Extraction and isolation of fungal metabolites

Fungal strains were grown on potato dextrose agar (PDA, Applichem, Darmstadt, Germany) at 25 °C for four weeks, and then the media containing fungal mycelium were cut in small pieces and extracted directly with ethyl acetate for 30 min. After filtration the solvent was evaporated to dryness under reduced pressure yielding a yellow crude extract (~100 mg). The extracts were re-dissolved in a mixture of DMSO/methanol/isopropyl alcohol (7:2:1) for a further purification by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Pure compounds were isolated from the crude extracts by semi-preparative RP-HPLC.

5.4. Bioassays

Bioassays to test for antiparasitic or cytotoxic activity of major compounds **5**, **19** and **20** were performed as described previously (Grundmann et al., 2014). Briefly, strains of the parasitic protozoa *T. brucei rhodesiense*, *T. cruzi*, *L. donovani* and *P. falciparum*, or rat myoskeletal fibroblasts (L6 cells) were inoculated in 96-well microtiter plates with appropriate growth media and incubated with serial dilutions of each compound. Half maximal inhibitory concentrations (IC_{50}) were calculated from the cell proliferation curves for each treatment. As positive controls, melarsoprol was used for *T. brucei rhodesiense*, benznidazole for *T. cruzi*, miltefosine for *L. donovani*, chloroquine for *P. falciparum*, and podophyllotoxin for L6 cells.

5.5. Phylogenetic analyses

We used BLAST (Altschul et al., 1990) to retrieve the 25 fungal ITS sequences from GenBank with the highest similarity to our strains, plus the 25 closest sequences belonging to fungal type strains. We aligned all sequences using MAFFT v7.123b (Katoh and Standley, 2013) with the G-INS-i algorithm, and then eliminated the poorly aligned regions in the multiple alignment using Gblocks v0.91b (Castresana, 2000) before further analysis. A first Neighbor-Joining (NJ) tree was calculated with the package ape v3.2 for R v3.0.2 (Paradis et al., 2004; R Core Team, 2013) using the Kimura two-parameter model and 1000 bootstrap replicates. A Maximum Likelihood (ML) phylogeny was obtained with RAxML v7.2.8 (Stamatakis, 2006), using a GTRGAMMA model of nucleotide substitution and 1000 bootstrap replicates. Sequences from *Capronia mansonii* CBS 101.67 (GenBank accession NR_121262; Müller et al., 1987) and *Capronia munkii* DAOM 216390 (NR_121263; Untereiner, 1995) were used as outgroup in both phylogenies, but were subsequently removed from the trees to better display relationships among other sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.08.006.

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Chapter 6: Maciá-Vicente JG, Glynou K, Piepenbring M. (2016). A new species of Exophiala associated with roots. *Mycol Prog* **15**: 1–12.

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

JGMV: 50%
MP: 40%
Promovierende KG: 10%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende KG: 15% isolation of fungal strains, molecular characterization of fungal strains,
JGMV: 50% isolation of fungal strains, molecular and morphological characterization of fungal strains, phylogenetic analyses, preparation and curation of voucher strains
MP: 35% morphological characterization of fungal strains

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende KG: 20% collection of ITS sequencing data and ecological data
JGMV: 50% collection of LSU, SSU, TEF, TUB and ACT sequencing data, retrieval of sequence data from public databases
MP: 30% obtainment of morphological data

(4) zur Analyse und Interpretation der Daten

JGMV: 50%
MP: 30%
Promovierende KG: 20%

(5) zum Verfassen des Manuskripts

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Zustimmende Bestätigungen der oben genannten Angaben:

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ORIGINAL ARTICLE

A new species of *Exophiala* associated with roots

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Abstract A new species of the genus *Exophiala* (Herpotrichiellaceae, Ascomycota), *Exophiala radicis*, is described. The description is based on five strains isolated as endophytes from roots of the brassicaceous plant *Microthlaspi perfoliatum* s.l., collected at different localities in Europe. As evidenced by phylogenetic analyses of regions of the ribosomal DNA [the small and large subunits, and the internal transcribed spacers (ITS)] and the translation elongation factor 1-α, the β-tubulin, and the actin genes, the new species is closely related to *Exophiala tremulae* and *Exophiala equina*. *E. radicis* differs from *E. tremulae* morphologically by the shape and size of their conidia. A comparison of ITS sequences of *E. radicis* with GenBank records suggests that the species has a wide distribution in the northern hemisphere, and that it is commonly associated with living plant roots, indicating potential adaptations to this substrate.

Keywords Chaetothyriales · Endophytes · Exophillic acid · Roots · *salmonis*-clade

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Introduction

The genus *Exophiala* constitutes a polymorphic group of ascomycetous fungi within the family Herpotrichiellaceae (Chaetothyriales). It includes dematiaceous anamorphic species characterized by annellidic conidiogenesis and frequent yeast-like states. Its known teleomorphs belong in the genus *Capronia* (Carmichael 1967; Hironaga et al. 1981; De Hoog et al. 2011). Most studies on *Exophiala* species focus on their importance as etiologic agents of disease in animals and humans (Richards et al. 1978; Zeng and de Hoog 2008; De Hoog et al. 2011; Najafzadeh et al. 2013; Wen et al. 2015), and they include assessments of their occurrence in anthropogenic habitats, such as bathroom facilities or bottled water, which constitute potential sources of infection (Iwatsu et al. 1991; Matos et al. 2002; Ávila et al. 2005; Isola et al. 2013). However, the pathogenic lifestyle of *Exophiala* species is opportunistic, and members of the genus are frequently isolated from natural environments independent of potential animal hosts, such as bulk soil, biological crusts, rock surfaces, air, natural water masses, the rhizosphere, and plant tissues (Addy et al. 2005; Julou et al. 2005; Bates et al. 2006; Neubert et al. 2006; Bukovská et al. 2010; De Hoog et al. 2011; Ferrari et al. 2011). Such diversity of ecological sources indicates that the species of *Exophiala* have versatile lifestyles with adaptations to thrive in multiple habitats. This is reflected in a pleomorphism in the genus, with species displaying synanamorphs (e.g., *Phaeococcomyces* or *Phialophora*; Untereiner et al. 1995; De Hoog et al. 2011) that include the production of budding cells (yeasts) by many species (Zeng and de Hoog 2008). The polymorphic nature of *Exophiala* species make them difficult to be identified by their morphology alone and, therefore, sequencing of nuclear regions of the ribosomal DNA is considered as necessary for identification of species (Untereiner and Naveau 1999; Bates et al. 2006; Zeng and de Hoog 2008; De Hoog et al. 2011).

In a recent study, the secondary metabolites produced in culture by five isolates of an *Exophiala* species were analyzed (Cheikh-Ali et al. 2015). The strains were isolated as endophytes from roots of *Microthlaspi perfoliatum* s.l. (L.) F.K. Meyer (Brassicaceae; Ali et al. 2016) growing at different localities in Europe, and all produced a set of natural products similar to exophillic acid, a metabolite previously described from *Exophiala pisciphila* McGinnis & Ajello (McGinnis and Ajello 1974; Ondeyka et al. 2003). Differences in their profiles of secondary metabolite production could be linked to morphological differences on their cultures: strains with flat and slimy colonies produced chemical derivatives containing a monosaccharide moiety (β-D-glucopyranosyl), while strains with dome-shaped colonies and aerial mycelium had similar derivatives but without the monosaccharide (Cheikh-Ali et al. 2015). In spite of their morphological and chemical differences, all strains are considered to pertain to the same species according to similarities in their micromorphology and internal transcribed spacer region (ITS) sequences. However, they could not be classified in any known species of *Exophiala* according to their morphological traits and phylogenetic affinities. Here, we provide a formal description of these strains based on morphological, molecular, and ecological data.

Materials and methods

Strains and culture conditions

The strains in this study were isolated in 2013 as root endophytes from several specimens of *Microthalspi perfoliatum* s.l. collected at different localities in Europe (Glynou et al. 2016). Strain P1095 was isolated from a plant in eastern France, strains P1860 and P1910 were isolated from individual plants within the same population in Bulgaria, and strains P2772 and P2854 originated from different plant populations in Germany. The reference ex-type strain of *Exophiala tremulae* W. Wang (CBS 129355; Crous et al. 2011) was obtained from the KNAW-CBS Fungal Biodiversity Centre (CBS). Strains are maintained on corn meal agar slants covered with mineral oil at room temperature at the IPF (Integrative Fungal Research) collection hosted at the Goethe University (Frankfurt am Main, Germany). Duplicate cultures are also deposited in the CBS culture collection (Utrecht, The Netherlands).

The strains were grown on 2 % malt extract agar (MEA, Applichem, Darmstadt, Germany) and potato dextrose agar (PDA, Applichem). They were cultured in triplicate for morphological examinations and growth measurements. For microscopic observations, samples were prepared as described in De Hoog et al. (2011) and observed after 7 to 14 days, or directly mounted in squash preparations from older cultures. Micrographs were taken on slides stained with lactophenol blue, using a Zeiss Axio Lab.A1 microscope and an Axiocam ERc 5 s camera (Zeiss, Hamburg, Germany). Microscopic structures were drawn from preparations mounted with distilled water and observed in a Zeiss Axioscop 2 plus, aided by a scale in the ocular and a scaled grid. Radial growth rates of colonies were measured periodically from cultures inoculated using mycelium on a 5-mm-diameter agar plug and incubated at either 25 °C or 37 °C.

DNA amplification and sequencing

Sequences of the ITS regions 1 and 2 and the 5.8S rDNA of the strains in this study are available in GenBank (Table 1; Glynou et al. 2016). We amplified and sequenced five additional nuclear loci in polymerase chain reactions containing 1 μL of DNA template, 2-mM $MgCl_2$, 0.2-mM dNTPs, 0.3 μM of each primer, and 0.5 U Taq polymerase (VWR International, Darmstadt, Germany). A part of the large subunit (LSU) of the rDNA was amplified using the primer pair LR0R/LR7 (Hopple and Vilgalys 1994) with the following temperature cycles: 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 48 °C for 45 s, and 72 °C for 2 min, and a final step of 72 °C for 5 min. The partial small subunit (SSU) of the rDNA was amplified with primers NSSU131/NS24 (Kauff and Lutzoni 2002) using the above cycling conditions, but with an annealing temperature of 52 °C. The partial translation elongation factor 1-α (*TEF1-α*) and β-tubulin (*β-tub*) genes were amplified with primer pairs Ef1-728F/Ef1-986R (Carbone and Kohn 1999) and Bt2a/Bt2b (Glass and Donaldson 1995), respectively, as in De Hoog et al. (2011). The partial actin gene (*act1*) was amplified with primers LPW17499/LPW17500 (Woo et al. 2013) with the following temperature cycles: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and one step of 72 °C for 5 min. Amplicons were purified with the EZNA Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced at GATC Biotech (Konstanz, Germany). Sequences of the SSU, *TEF1-α*, *β-tub*, and *act1* were likewise obtained for the reference strain *E. tremulae* CBS 129355. All sequences generated in this work have been deposited in GenBank (see Table 1 for accession numbers).

Phylogenetic analyses

Phylogenetic analyses were performed separately for every locus using maximum likelihood (ML) and Bayesian phylogenetic inference. A selection of available representative sequences from strains of the closest *Exophiala* species within the *salmonis* clade of *Exophiala* (De Hoog et al. 2011) was included as a reference (Table 1). Sequences were aligned using the G-INS-i algorithm of MAFFT v7.123b (Katoh and Standley 2013) and then trimmed with Gblocks v0.91b (Castresana 2000). For ML phylogenies, we used RAxML

Table 1 Reference strains of *Exophiala* included in the phylogenetic analyses

Proposed classification[a]	Original name	Strain[b]	Source	Location	GenBank accessions						Reference
					ITS	LSU	SSU	*TEF1-α*	*β-tub*	*act1*	
Exophiala aquamarina	*Exophiala aquamarina*	CBS 119918 (T)	Skin of leafy sea dragon	USA	NR_111626	-	JN856012	-	JN112434	JN112388	De Hoog et al. (2011)
Exophiala brunnea	*Exophiala brunnea*	CBS 587.66 (T)	Litter of *Acacia karoo*	South Africa	NR_119959	-	JN856013	JN128783	JN112442	JN112393	De Hoog et al. (2011)
Exophiala cancerae	*Exophiala cancerae*	CBS 120420 (T)	Diseased mangrove crab	Brazil	HQ659023	-	-	JN128800	JN112444	JN112394	De Hoog et al. (2011)
Exophiala equina	*Exophiala equina*	CBS 116009	Lung tissue of Galapagos tortoise	USA	KF928433	KF928497	KF155200	JN128805	KF928561	-	De Hoog et al. (2011)
Exophiala equina	*Exophiala equina*	CBS 119.23 (T)	Subcutaneous infection of horse	Italy	NR_111627	-	JN856017	JN128814	JN112462	JN112401	Pollacci (1923)
Exophiala equina	*Exophiala equina*	CBS 128222	Human	South Korea	KF928432	KF928496	-	-	JQ797585	-	Najafzadeh et al. (2013)
Exophiala pisciphila	*Exophiala pisciphila*	CBS 119914	Potbelly seahorse	unknown	JF747133	-	-	JN128791	JN112495	-	De Hoog et al. (2011)
Exophiala pisciphila	*Exophiala pisciphila*	CBS 121500	Human nail	Germany	JF747134	-	-	JN128789	JN112496	JN112414	De Hoog et al. (2011)
Exophiala pisciphila	*Exophiala pisciphila*	CBS 537.73 (T)	Catfish	USA	NR_121269	AF361052	JN856018	JN128788	JN112493	JN112412	McGinnis and Ajello (1974)
Exophiala psychrophila	*Exophiala psychrophila*	CBS 191.87 (T)	Salmon in fish farm	Norway	JF747135	-	JN856019	JN128798	JN112497	-	Pedersen and Landvag (1989)
Exophiala radicis	*Exophiala* sp.	CBS 120387	Human, toe nail	Denmark	JF747100	-	-	JN128822	JN112466	JN112403	De Hoog et al. (2011)
Exophiala radicis	*Exophiala* sp.	CBS 121502	*Olea* twig	Italy	JF747106	-	-	JN128826	JN112472	JN112405	De Hoog et al. (2011)
Exophiala radicis	*Exophiala* sp.	CBS 121504	Tinea on leg of child (18 mo)	Germany	JF747098	-	-	JN128820	JN112464	JN112402	De Hoog et al. (2011)
Exophiala radicis	*Exophiala equina*	CBS 122263	Female age 56, foot	Denmark	KF928431	KF928495	-	JN128821	JN112465	-	De Hoog et al. (2011)
Exophiala radicis	*Exophiala equina*	CBS 160.89	Plant roots	The Netherlands	KF928430	KF928494	-	JN128824	JN112470	-	De Hoog et al. (2011)
Exophiala radicis	*Exophiala* sp.	CBS 661.76	Nematode cyst (*Heterodera*)	Germany	JF747103	-	-	JN128823	JN112469	JN112404	De Hoog et al. (2011)
Exophiala radicis	*Exophiala radicis*	P1095	Root endophyte of *Microthlaspi erraticum*	France	KT099195	**KT723444**	**KT723449**	**KT723454**	**KT723459**	**KT723439**	Glynou et al. (2016)
Exophiala radicis	*Exophiala radicis*	P1860	Root endophyte of *Microthlaspi erraticum*	Bulgaria	KT099197	**KT723445**	**KT723450**	**KT723455**	**KT723460**	**KT723440**	Glynou et al. (2016)

Table 1 (continued)

Proposed classification[a]	Original name	Strain[b]	Source	Location	GenBank accessions						Reference
					ITS	LSU	SSU	*TEF1-α*	*β-tub*	*act1*	
Exophiala radicis	*Exophiala radicis*	P1910	Root endophyte of *Microthlaspi erraticum*	Bulgaria	KT099198	**KT723446**	**KT723451**	**KT723456**	**KT723461**	**KT723441**	Glynou et al. (2016)
Exophiala radicis	*Exophiala radicis*	P2772	Root endophyte of *Microthlaspi erraticum*	Germany	KT099203	**KT723447**	**KT723452**	**KT723457**	**KT723462**	**KT723442**	Glynou et al. (2016)
Exophiala radicis	*Exophiala radicis*	P2854 (T)	Root endophyte of *Microthlaspi perfoliatum*	Germany	KT099204	**KT723448**	**KT723453**	**KT723458**	**KT723463**	**KT723443**	Glynou et al. (2016)
Exophiala salmonis	*Exophiala salmonis*	CBS 110371	Drinking water	The Netherlands	JF747139	KF155179	KF155198	JN128748	JN112501	JN112417	De Hoog et al. (2011)
Exophiala salmonis	*Exophiala salmonis*	CBS 120274	Water	The Netherlands	KF928434	KF928498	-	JN128802	JN112500	JN112416	De Hoog et al. (2011)
Exophiala salmonis	*Exophiala salmonis*	CBS 157.67 (T)	Cerebral mycetoma of salmon	Canada	NR_121270	AY213702	JN856020	JN128747	JN112499	JN112415	Carmichael (1967)
Exophiala tremulae	*Exophiala* sp.	CBS 122270	Human foot	unknown	JF747101	-	-	JN128818	JN112467	-	De Hoog et al. (2011)
Exophiala tremulae	*Exophiala tremulae*	CBS 129355 (T)	Roots of *Populus tremula*	Canada	FJ665274	JF951155	**KT894147**	**KT894149**	**KT894148**	**KT894146**	Crous et al. (2011)

GenBank accession numbers of sequences generated in this study are shown in bold.

[a] Proposed identification according to phylogenetic results in this study.

[b] (T) indicates type strains.

v8.0.0 (Stamatakis 2014) with the general time reversible model of nucleotide substitution and the Γ model of rate heterogeneity (GTRGAMMA), and 1000 bootstrap replicates. For Bayesian analyses, we used MrBayes v3.2.2 × 64 (Ronquist et al. 2012) with the GTRGAMMA model and two independent MCMC runs for 10 M generations with sampling every 100th generation, and a burn-in of 30 % of the sampled trees. Genealogical concordance was assessed visually and with the partition homogeneity test as implemented in PAUP* v4.0a146 (Farris et al. 1995; Swofford 2003). A multilocus tree containing representatives of all species was constructed with sequences for the ITS, *TEF1-α*, and *β-tub* regions following the same procedure described above, but using a burn-in of 50 % in the Bayesian analysis. We excluded *act1* from the multilocus analysis because it yielded a tree topology that differs from that obtained for the other loci (Fig. S1), and because of the lack of sequences for several strains (Table 1). The calculated trees were edited using the package APE v3.2 for R v3.0.2 (Paradis et al. 2004; R Core Team 2013).

A second set of analyses was performed to infer the ecological and geographic occurrence of the species. We used BLAST (Altschul et al. 1990) to retrieve from GenBank the 250 closest ITS sequences to each of our strains, and to those of the closest type strains, *E. tremulae* CBS 129355 (FJ665274) and *Exophiala equina* (Pollacci) De Hoog, Vicente, Najafzadeh, Harrak, Badali & Seyedmousavi (strain CBS 119.23; NR_111627). We selected sequences that in a preliminary neighbor-joining (NJ) analysis clustered with those used as BLAST queries, and not with other *Exophiala* species (e.g., *E. pisciphila* or *Exophiala salmonis* J.W. Carmich.). We also removed an additional 17 sequences, mostly originating from environmental sequencing, because they resulted in low-quality alignments and long branches. The final selection included 103 GenBank records, for which we retrieved information on their isolation source and geographic origin from GenBank. When such information was absent in the database, we searched for it in the corresponding bibliographic references when available, or contacted the corresponding authors. Sequences were aligned and trimmed as described above, and then a NJ tree was calculated with APE using the Kimura two-parameter model and 1000 bootstrap replicates. Sequences from *E. salmonis* CBS 157.67 (NR_121270), *Exophiala aquamarina* De Hoog, Vicente, Najafzadeh, Harrak, Badali, Seyedmousavi & Nyaoke CBS 119918 (NR_111626), and *E. pisciphila* CBS 537.73 (NR_121269) were used as the outgroup. Additional support for this tree was obtained as bootstrap scores in an ML analysis as previously described. For each cluster, we calculated the proportion of records belonging to the following source categories: i) animal/human, when obtained from diseased animals/humans or lesions, ii) human-related, when obtained from anthropogenic substrates (e.g., bathroom items, bottled water or washing machines), iii) plant, when directly isolated from a plant organ, iv) soil, including the rhizosphere of plants, or v) others, including other sources such as water from lakes, or fungal fruiting bodies. We also calculated the proportion of records detected at different geographical localities, grouped by continent. Details of the sequences included in this analysis are provided in Supplementary Table S1. All alignments have been deposited in TreeBASE under accession number S18495.

Results

Phylogeny

Comparisons of the ITS sequences of the five *Exophiala* strains herein studied with those in the GenBank nucleotide database using BLAST show that they have a high similarity to *E. tremulae* (99 % identity) and *E. equina* (99 % identity), with the next closest species being *E. salmonis* (96 % identity) and *E. pisciphila* (94 % identity). The sequences of the five strains cluster together in all phylogenetic trees calculated, both for each individual locus (Figs. 1a and S1) and in a multilocus analysis with concatenated ITS, *TEF1-α*, and *β-tub* sequence partitions (Fig. 1b). In both phylogenies, the sequences of our *Exophiala* strains are placed in close proximity to *E. tremulae* and *E. equina* (Fig. 1). The grouping of these clusters is strongly supported in the ML analysis of the ITS alone (Fig. 1a), and in both the ML and the Bayesian analyses using three independent loci (Fig. 1b). The latter also shows that strains P1095 and P2854 constitute a clade slightly, but significantly, separated from the other strains in this work (Fig. 1b). Other non-type reference isolates included in the phylogenetic study, which had been identified as *E. equina* or *Exophiala* sp., grouped with the isolates from *Microthlaspi*. Based on these results we propose a new species, *Exophiala radicis*, based on isolates in that clade (Table 1). In addition, a new classification of strain *Exophiala* sp. CBS 122270 as *E. tremulae* is proposed based on its grouping alongside the type specimen of this species (Fig. 1).

Morphology

We compared the morphology of strain *E. radicis* P2854 with that of the type culture of its closest described species, *E. tremulae* CBS 129355 (Fig. 4). Whereas both strains have similar rates of radial growth on both MEA (0.8–0.9 and 0.7–0.9 mm day^{-1}, respectively) and PDA (0.8–0.9 and 0.9–1 mm day^{-1}), their colony morphologies differ clearly. *E. radicis* P2854 produces greyish colonies, which are velvety due to the production of floccose aerial mycelium (Fig. 2a, b). On the other hand, *E. tremulae* has darker greyish colonies, flat, with humped aerial mycelium (Fig. 2c, d). The colony morphology of *E. tremulae* is, however, similar to that of

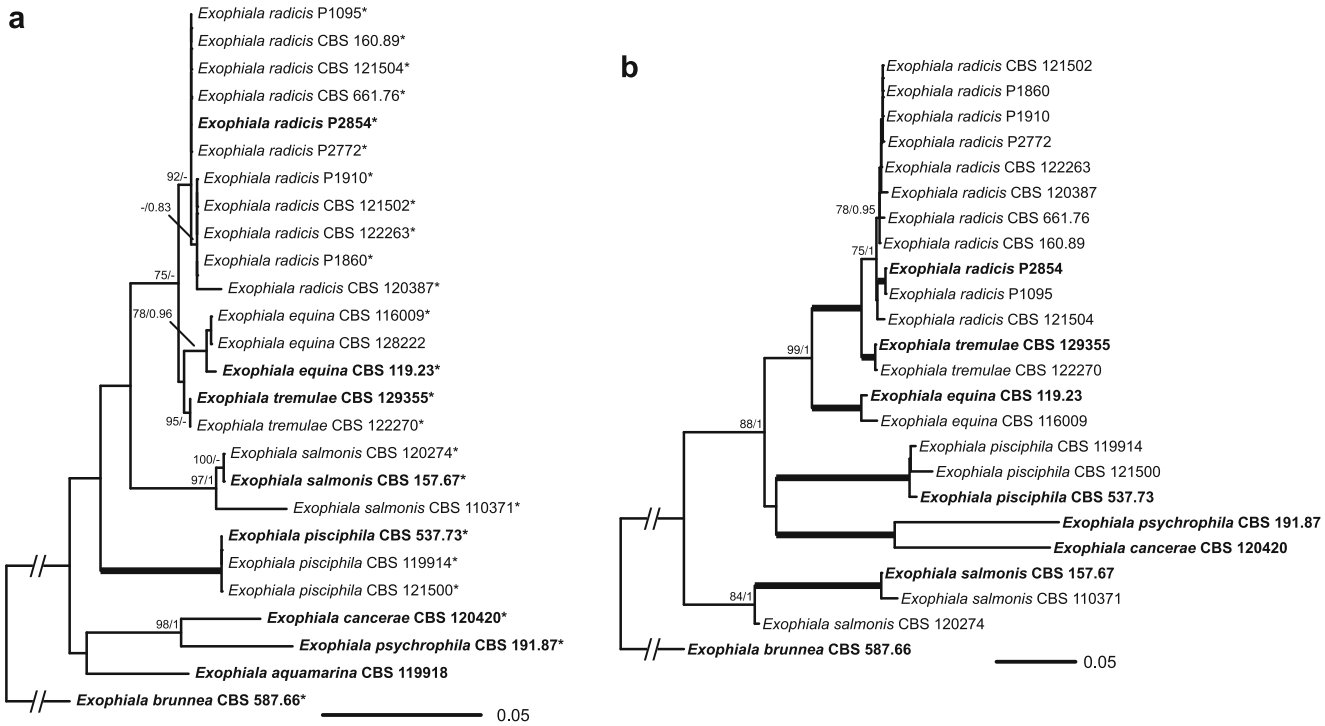

Fig. 1 Phylogenetic relationships of the strains of this study and other *Exophiala* species within the *salmonis* clade (De Hoog et al. 2011). Trees represent topologies based on the ML analysis of the ITS rDNA sequences (**a**) and of a concatenated alignment containing ITS, *TEF1-α*, and *β-tub* sequences (**b**). Node support values correspond to bootstrap analyses based on 1000 replicates for the ML analysis, and posterior probabilities (PP) as obtained by Bayesian inference (ML/PP). Only support ML/PP values equal of higher than 70 % and 0.8 are displayed for each node, respectively. Thickened branches indicate a strong combined support of ML = 100 % and PP = 1. Sequences highlighted in bold were obtained from type material, and asterisks in **a** indicate strains included in the multilocus analysis (**b**). Both trees were rooted with *Exophiala brunnea* CBS 587.66. Notches in root outgroup branches indicate that these have been shortened manually. *Bars* represent substitutions per nucleotide position

strains *E. radicis* P1860, P1910 and P2772, which have been previously described to produce flat colonies as opposed to P2854 and P1095 (Cheikh-Ali et al. 2015). Microscopic differences between both strains are most evident by the shape and size of their conidia. *E. radicis* P2854 first grows as sterile mycelium, but after ten days it can also produce abundant, aseptate, ellipsoidal to allantoid conidia, with a discernible scar at the base, with a size of 2–3 × (4–)5–6.5(−9) μm (Figs. 2f–j, 3). *E. tremulae* produces abundant conidia after 7 days of growth, which are mostly aseptate, cylindrical with rounded apex and a scar at the base, with a size of 2.5–3.5(−4) × 7–11(−13) μm (Figs. 2k–m, 4). Our measurements of the size of conidia of *E. tremulae* disagree with data in the original description of the species by Crous et al. (2011) that is based on the same strain. The latter reported a conidia size of 1–1.5 × 3–4 μm, being roughly half of what we observed.

Ecology and distribution

For a comparative analysis of the ecology and geographic origins of strains closely related to *E. radicis*, we constructed a distance-based tree containing the ITS sequences from our strains, the type strains of the closest species (*E. tremulae* and *E. equina*), and all their closest BLAST matches. Most sequences cluster in four major groups (Fig. 5) corresponding to sequences similar to *E. radicis*, *E. tremulae*, and two groups containing entries identified as *E. equina*, which were termed *E. equina* and *E.* cf. *equina* (Fig. 5). The cluster containing *E. radicis* comprises the largest number of BLAST records. The group *E.* cf. *equina*, with a bootstrap support value of 72 % in the ML analysis, is mostly constituted by sequences from uncultured soil fungi from a single unpublished study (50 % of the sequences), plus a few other sequences from strains obtained from different sources and mostly named *E. equina*.

The majority of sequences clustering alongside *E. radicis* and *E. tremulae* originate from fungi associated with plants or soil, including rhizospheric soil (Fig. 5). Approximately 95 % of the records from plant material originate from roots (all but three, two of which have unknown origin; Table S1) and, in most cases, from healthy plants. These groups also have similar geographic origins, with most of their records originating from Europe and North America, and to a lesser extent from Asia. Records from *E. equina* differ from the previous ones by their greater occurrence in human-related substrata. The

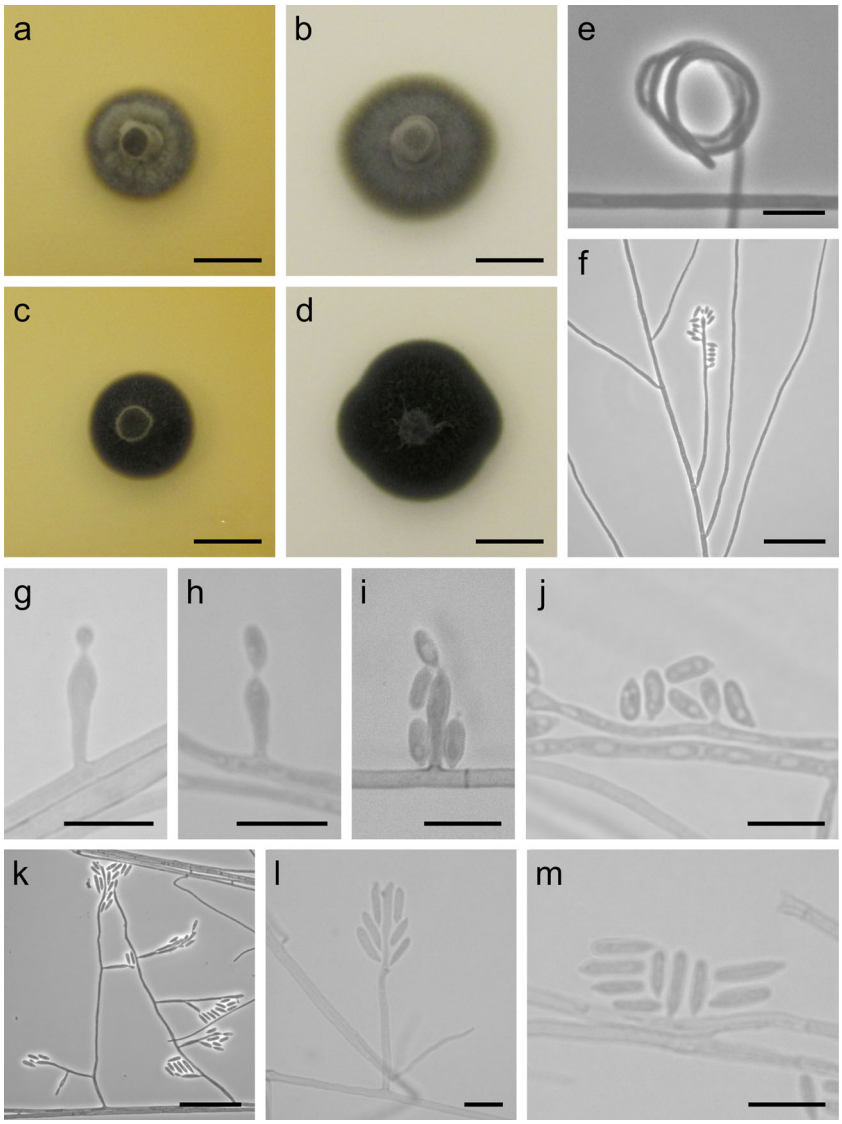

Fig. 2 *Exophiala radicis* P2854 (**a**, **b**, **e**–**j**) and *Exophiala tremulae* CBS 129355 (**c**, **d**, **k**–**m**). **a**, Colony of *E. radicis* on MEA. **b**, Colony of *E. radicis* on PDA. **c**, Colony of *E. tremulae* on MEA. **d**, Colony of *E. tremulae* on PDA. **e**, Hyphal coil produced by *E. radicis*. **c**–**i**, Conidiogenous cells of *E. radicis*. **j**, Conidia of *E. radicis*. **k**–**l**, Conidiogenous cells of *E. tremulae*. **m**, Conidia of *E. tremulae*. *Scale bars*: **a**–**d** = 10 mm; **e**, **g**–**j**, **l**, **m** = 10 μm; **f**, **k** = 40 μm

majority of them also originate from Europe, although they show a broader distribution than *E. radicis* and *E. tremulae*, and they include the only observations from South America (Fig. 5). All groups have a rather similar proportion of isolates originating from animals or humans.

Taxonomy

***Exophiala radicis* Maciá-Vicente, Glynou & M. Piepenbr, sp. nov**. — Figs. 1, 2, and 3, S1.

MycoBank: MB815365.

ITS barcode: KT099204.

Alternative markers: LSU = KT723448; SSU = KT723453; *TEF1-α* = KT723458; *β-tub* = KT723463; *act1* = KT723443.

Etymology: Named after the plant organ (roots) from where the species has been repeatedly isolated.

Diagnosis Colonies were slow-growing, circular, either felty, greyish black, or slimy and more or less velvety, black. Reverse was grayish black. No diffusible pigments were observed. No yeast growth was observed. Radial growth rates at 25 °C were 0.8–0.9 mm day^{-1} on MEA, and 0.8–0.9 mm day^{-1} on PDA. No growth was observed at 37 °C for up to three weeks, nor after a subsequent incubation at 25 °C.

Morphological description *Vegetative hyphae* were unswollen with a width of 2.5–4 μm, with lateral branches originating close to septa, rarely fusing by anastomosis, forming fascicles protruding from black slimy structures in old colonies. Other hyphae were torulose, up to 5 μm wide, forming lateral ramifications by budding. Hyphae were hyaline to light brownish, broader (older?) hyphae being more strongly pigmented. Hyphal coils were rarely observed, with spirals of 32 or 39-μm diam. that were seen twice in one of

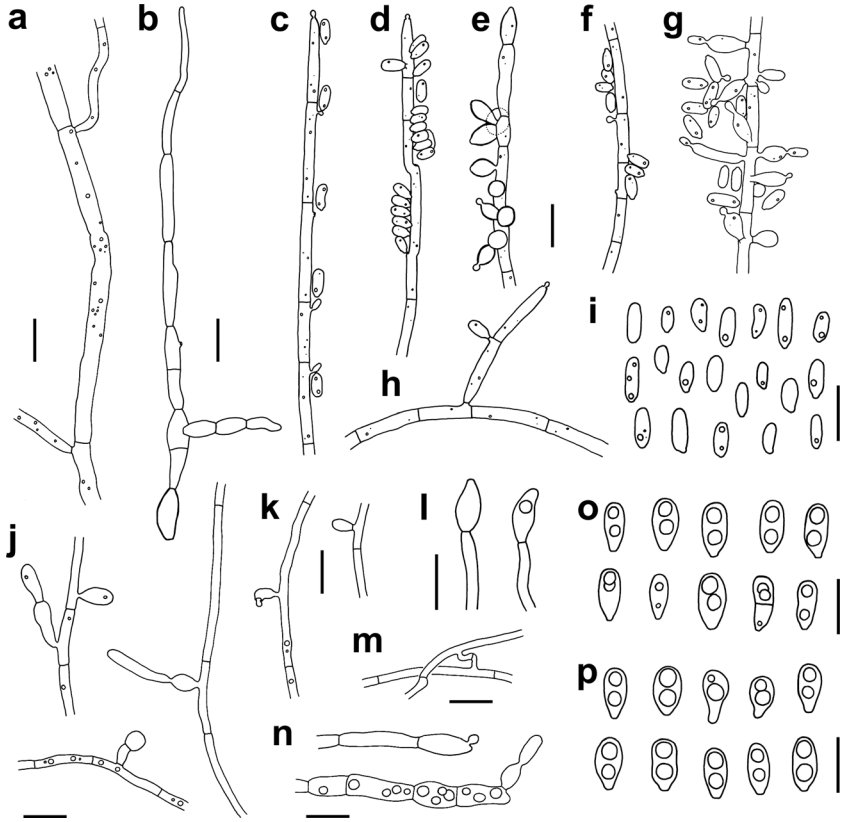

Fig. 3 Microscopic structures of *Exophiala radicis*. **a**, Vegetative hypha with lateral ramifications (P1910, after 19 days). **b**, Torulose hypha (P1910, after 19 days). **c**–**i**, Hyphae with terminal, intercalary, and/or lateral conidiogenous cells and conidia (P2854, after 11 days). **c**, Sporogenous tip of a hypha with one apical and several intercalary conidiogenous cells. **d**, Sporogenous tip of a hypha with sympodial growth of conidiogenous hypha. **e**, Tip of a hypha with lateral conidiogenous cells. **f**, Intercalary hyphal cells forming conidia. **g**, Intercalary hyphal cells that formed conidia and conidiogenous cells. **h**, Hypha with lateral conidiogenous hyphal cells. **i**, Primary conidia. **j**, Hyphae with lateral conidiogenous cells that mostly develop lateral hyphae (P2854, after 32 days). **k**, Hyphae with lateral conidiogenous cells (P1910, after 5 months). **l**, Hyphae with conidiogenous loci and young conidia at their tips (P1910, after 5 months). **m**, Two hyphae with a conjugation bridge (P1910, after 5 months). **n**, Torulose hyphae (P2854, after 4 months and 22 days). **o**, Conidia (P1910, after 5 months). **p**, Conidia (P2854, after 4 months and 22 days). *Scale bars* = 10 μm

approx. 20 preparations. In 10 to 11 days old cultures, many hyphae were sterile, but other hyphae exhibited diverse *conidiogenous structures* forming primary conidia (Fig. 3c–h): The distal parts of some hyphae were formed by conidiogenous hyphal cells with one conidiogenous locus each, located at the tip of the distal cell or close to the distal septum of intercalary cells. At each conidiogenous locus, several conidia were produced successively, with up to nine conidia observed for one conidiogenous locus. Some conidiogenous terminal cells reassumed hyphal growth sympodially, resulting in geniculate hyphae. At other hyphal tips, lateral, ovoid to clavate conidiogenous cells were observed. Some intercalary hyphal cells presented conidiogenous loci at any place or produced lateral ovoid to clavate or hyphal conidiogenous cells. *Lateral*, *ovoid to clavate conidiogenous cells* were pale brown to subhyaline, of lateral origin independent of septa, 2.5–4(−5) × (5–)7–14(−18) μm (n = 15), sometimes with walls that were thicker than those of other cells, each with one apical locus, sometimes developing torulose hyphae instead of conidia. Colonies older than one month produced secondary conidia abundantly from undifferentiated conidiophores. *Primary conidia* were aseptate, hyaline, ellipsoidal, cylindrical, or allantoid, 2–3 × (4–)5–6.5(−9) μm (n = 40), with a conspicuous scar approx. 1 μm wide at the base, with smooth walls, containing no evident or few, small oil drops. *Secondary conidia* were mostly aseptate, pale brown, rarely with one transverse septum, ellipsoidal, ovoid to slightly clavate, (3–)3.5–5 × (7–)8–10(−11) μm (n = 40), with a conspicuous scar 1–1.5(−2) μm wide at the base. Large (old) conidia had a thickened, light brown, smooth wall, and most contained two oil drops. *Chlamydospores* were not observed.

Holotype Germany, Baden-Württemberg, 49.54 N, 9.34 E, alt. approx. 415 m above sea level (m a.s.l.), endophytic in roots of *Microthlaspi perfoliatum* s. str. (L.) F.K. Meyer (Brassicaceae), 6 June 2013, collected by K Glynou and JG Maciá-Vicente, isolated by K Glynou, FR 0219456 (ex-type cultures: IPF collection P2854 = CBS 140402).

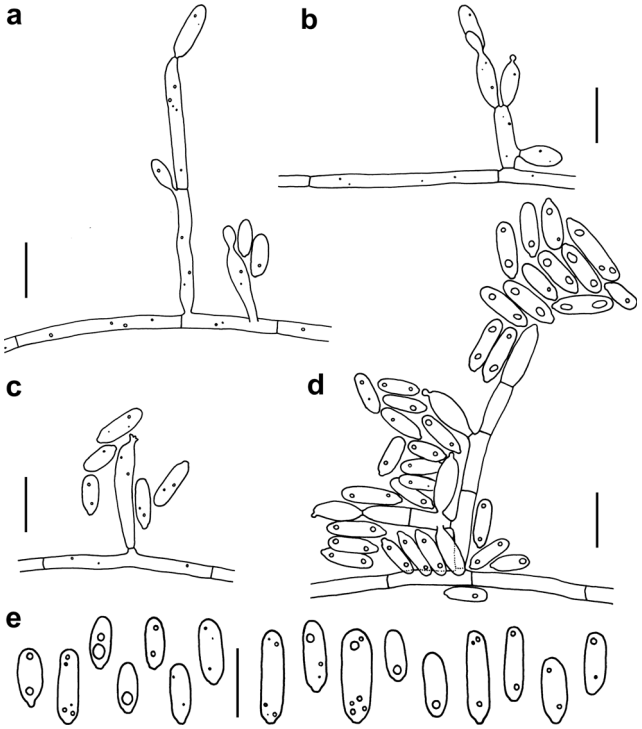

Fig. 4 Microscopic structures of *Exophiala tremulae*. **a**–**d**, Hyphae with conidiogenous cells and conidia. **e**, Conidia. *Scale bars* = 10 μm

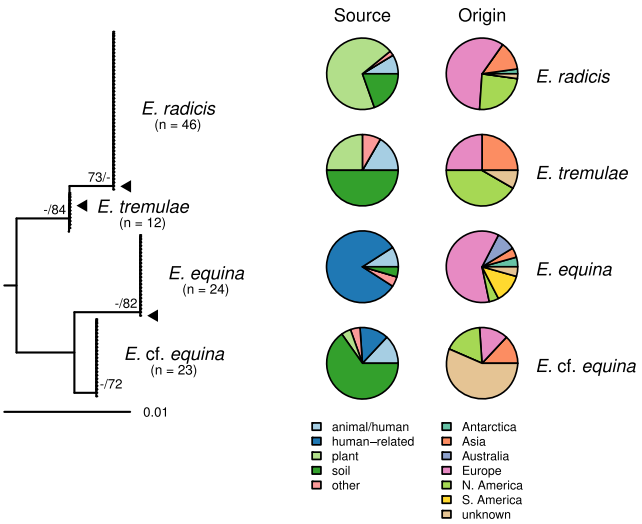

Fig. 5 Isolation sources and geographic origins of the strains of this study, the related type strains for *E. tremulae* and *E. equina*, and their closest GenBank records based on partial ITS rDNA similarity. On the left, a neighbor-joining distance tree with bootstrap support values (NJ/ML) of all sequences selected is shown. The outgroup is omitted without modifying the tree topology. The *bar* represents substitutions per nucleotide position. Four major groups of sequences defined by sequence similarity of approximately 100 %, are labeled as *E. radicis*, *E. tremulae*, *E. equina* and *E.* cf. *equina*. *Arrowheads* indicate the position of sequences from the type strains of each species, and *n* indicates the number of sequences within each cluster. On the *right side*, *pie charts* represent the proportion of sequences within each group originating from different source categories or geographic regions

Additional cultures examined Germany, Franconia, 49.68 N, 10.00 E, alt. approx. 278 m a.s.l., endophytic in roots of *Microthlaspi erraticum* (Jordan) Tahir Ali et Thines, 6 June 2013, collected by K Glynou and JG Maciá-Vicente, isolated by K Glynou, IPF collection P2772 = CBS 140401. Bulgaria, Pernik, 42.70 N, 22.83 E, alt. approx. 770 m a.s.l., endophytic in roots of *M. erraticum*, 14 May 2013, collected by T Ali and S Ploch, isolated by K Glynou, IPF collection P1910 = CBS 140400. Bulgaria, Pernik, 42.70 N, 22.83 E, alt. approx. 770 m a.s.l., endophytic in roots of *M. erraticum*, 14 May 2013, collected by T Ali and S Ploch, isolated by K Glynou, IPF collection P1860 = CBS 140399. France, Franche-Comté, 47.41 N, 6.56 E, alt. approx. 285 m a.s.l., endophytic in roots of *M. erraticum*, 19 May 2013, collected and isolated by JG Maciá-Vicente, IPF collection P1095 = CBS 140398. Ex-type of *Exophiala tremulae*, Canada, Alberta, Edmonton, Lamont, 664 m a.s.l., from roots of *Populus tremuloides*, CBS 129355.

Discussion

Morphological, phylogenetic, and ecological characteristics differ significantly between the strains herein studied and other described species of *Exophiala*, thus supporting the proposal of a new species to accommodate them. Morphological examination of the strains sustain the separation of *E. radicis* from its closest relatives, *E. tremulae* and *E. equina*. Both *E. tremulae* and *E. equina* have been recently described (Crous et al. 2011; De Hoog et al. 2011). The latter was named after a morphological and phylogenetic revision of *Haplographium debellae-marengoi* var. *equinum*, first isolated in 1923 in Italy, from a subcutaneous infection of the lower leg of a horse (Pollacci 1923; De Hoog et al. 2011). *Exophiala tremulae* was described from a strain isolated from roots of *Populus tremuloides* seedlings in Canada (Crous et al. 2011), and up to now it was only represented by the type specimen CBS 129355. Although *E. tremulae* was first regarded as a synonym of *E. equina* (De Hoog et al. 2011), it is now recognized as an independent species. *E. radicis* differs from *E. tremulae*, its closest species, by smaller and mostly ellipsoidal primary conidia produced in about 10-day-old cultures. At the same time, conidia formed by *E. tremulae* are larger and cylindrical. Other characteristics of *E. radicis* are consistent with features of the genus *Exophiala* (De Hoog and Hermanides-Nijhof 1977), but we did not observe production of yeast cells, not even in cultures developing a slimy consistency that is characteristic of yeast-forming colonies. Absence of yeast cells is common in species within the *salmonis* clade like *E. salmonis* and *E. pisciphila*, which differentiates them from other *Exophiala* species that are often referred to as black yeasts, together with other fungal taxa with a polyphyletic origin (Sterflinger 2006; Zeng and de Hoog 2008). At

conidiogenous loci of some conidiogenous cells, a periclinal thickening was observed, but no annellidic proliferation.

The phylogenetic analyses support the separation of *E. radicis* from *E. tremulae* and *E. equina*. Molecular data clearly placed the strains within the clade *salmonis* of the genus, defined by De Hoog et al. (2011) based on SSU sequence data. This clade contains waterborne species of *Exophiala* that are mesophylic or psychrotolerant, and that can be found causing disease most commonly on cold-blooded animals. Other species such as those in clade *dermatitidis* are more frequent on warm blooded animals (Haase et al. 1999; De Hoog et al. 2011). The ITS rDNA region is useful for species delimitation in *Exophiala*, and its sequencing is regarded as necessary for identification due to the high morphological variability of species in this genus (Uijthof and de Hoog 1995; Zeng and de Hoog 2008; De Hoog et al. 2011). However, an ITS-based phylogeny by De Hoog et al. (2011) showed that a clade containing different strains of *E. equina* presented lower support values than others from sister species. In the same study, a multilocus analysis established two well-supported groups within *E. equina*, indicating a considerable variability within this species (De Hoog et al. 2011). This could explain the resemblance in our analyses of two strains identified as *E. equina* (CBS 180.89 and CBS 122263) to our strains, while other strains of the same species were rather different (CBS 128222 and CBS 116009). The same clustering pattern for these strains has been previously shown based on a phylogenetic analysis with ITS, LSU, and *β-tub* sequences (Attili-Angelis et al. 2014). Moreover, De Hoog et al. (2011) demonstrated that strains CBS 160.89 and CBS 122263 are rather different from the *E. equina* type strain CBS 119.23, while strain CBS 116009 is closely related to it. This supports the differences between our strains and the type of *E. equina* herein observed.

A recent analysis of the secondary metabolites produced by the *E. radicis* isolates studied here showed similarities with other species within the *salmonis* clade (Cheikh-Ali et al. 2015). In this work, all strains produced exophillic acid or similar compounds, which have been previously described only from *E. pisciphila* (Ondeyka et al. 2003). This could indicate that production of exophillic acid and its derivatives is a synapomorphic trait for this clade, although it has not been identified in some of its members like *E. salmonis* or *E. equina*. A screening for this compound in species related to *E. radicis* and *E. pisciphila* would help to evaluate the suitability of these secondary metabolites as chemotaxonomic markers for this group, as has been established for other compounds and fungi (Frisvad et al. 2008; Surup et al. 2014). Interestingly, our multilocus phylogenetic analysis placed strains P1095 and P2854 in a clade slightly apart from other *E. radicis* strains. Such separation had been already observed by Cheikh-Ali et al. (2015) based on colony characteristics and chemical profiles, but it had not been evident by ITS sequence similarity alone. The main differences between both groups are that P1095 and P2854 form dry colonies with abundant aerial mycelium, and exophillic acid-like compounds lacking a β-D-glucopyranosyl moiety in their structure. Conversely, strains P1860, P1910, and P2772 have slimy colonies mostly without aerial mycelium, and produce exophillic acids and other derivatives containing the monosaccharide moiety. While Cheikh-Ali et al. (2015) could not attribute these differences to either genetically divergent populations or to the known pleomorphic nature of *Exophiala* (Untereiner and Naveau 1999; Wen et al. 2015), our new results hint towards the first possibility. Further study of the molecular differences among these strains is necessary to conclude whether they represent different cryptic species, as their morphological and chemical traits—but not their ITS sequences—suggest. Strains P1095 and P2854 were isolated from the Western-most localities in Europe, suggesting a biogeographic pattern in the distribution of *E. radicis* populations. This hypothesis, however, should be considered cautiously due to the low number of strains analyzed, and to the geographically close origins of the divergent strains P2772 and P2854 (ca. 50 Km apart).

Comparison of ITS sequences from our strains with sequences available in GenBank revealed that they are by no means the first records for this species. A multitude of highly similar sequences are available, which in most cases are only annotated to the genus level, and in a few cases, as *E. equina* or *E. salmonis*. Specimens that yielded sequence data similar to *E. radicis* were mostly obtained from roots of healthy plants, and often they were reported as root endophytes. Several species of *Exophiala* have been frequently isolated as endophytes, although they seldom represent important components of endophytic communities (Addy et al. 2005). Moreover, a few endophytic *Exophiala* strains have shown the ability to enhance growth and to confer certain tolerance to their hosts towards stressful conditions (Khan et al. 2011a, b), suggesting specific mechanisms of interaction with plants. While isolation sources similar to those for *E. radicis* were found for records close to *E. tremulae*, entries identical to *E. equina* mostly originate from human-related sources. This might explain the fact that *E. equina* is more widely distributed than the other two species, being *E. equina* the only one reported from South American and Australian sources. *E. radicis* and *E. tremulae* records appear to be largely restricted to the northern hemisphere, with the exception of one record for *E. radicis* in Antarctica. Interestingly, all groups defined here display a similar proportion of records from animals sources, indicating that the ability to infect and develop disease is a widespread characteristic of this group, and that these species share mechanisms for infection, as proposed by De Hoog et al. (2011). This analysis is certainly not exhaustive, and most probably reflects biases in the number of samplings/reports at different geographical regions, as evidenced by the

absence of samples from Africa. However, our data suggest hypotheses for particular ecological preferences and distribution of these species. Further samplings and identifications are necessary to better understand the natural reservoirs of these etiologic agents of disease in humans and other animals.

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Education

May 2013 – October 2016	**PhD fellowship in Biology** Department of Biological Sciences (Institute of Ecology, Evolution and Diversity) - Goethe University Frankfurt am Main Under the supervision of Prof. Dr. Marco Thines and Dr. Jose G. Maciá-Vicente PhD position framed within the Loewe excellence cluster "Integrative Fungal Research" (IPF)

Topics I covered during my PhD:

- Generation and molecular characterization of a collection of 2500 fungi
- Chemical analysis of ca. 1000 fungal strains
- Population genetics

I developed skills in:

- Next Generation Sequencing techniques (Illumina seq.) and molecular methods (DNA extraction, PCR, AFLP profiling)
- Physiology and biochemistry of microorganisms, isolation and curation of cultures
- Advanced statistical methods - Analysis of NGS data - Microsoft Office - R language
- Scientific writing - effective presentation and communication of results
- Working in multidisciplinary projects and collaborations

October 2011 – September 2012	**MSc Environmental Management** – Pass with Distinction Department of Agriculture and Environmental Sciences - University of Reading (UK)

Topics covered during my Master:

- Environmental Management – Principles and Practice
- Contaminated Land Management
- Practical Site Investigation and Assessment
- Remediation
- Environment and Development: Problems and Policies
- EU Environmental Law
- Resource and Environmental Economics
- Entrepreneurship and Business Skills

- Transport processes in the Environment
- Soils, Waste and Environmental Management

- MSc Dissertation: **"Mycorrhizal responses to commercial biochar addition to soil"**
  Under the supervision of Dr. Liz Shaw

I developed skills in:

- Greenhouse experiments
- Analysis of physicochemical soil properties
- Physiology of mycorrhizal fungi

November 2010 – March 2011	**Internship** Department of Crop Sciences – Agricultural University of Athens (Greece)

Topics covered:

- Extraction of secondary metabolites from the fungus *Drechslera avenae* with phytotoxic effects

October 2006 – June 2011	**BSc Biology** Department of Biology - University of Crete (Greece)

- Diploma Thesis: "Study of the response of lichens' mitochondria to abiotic stress with the use of fluorescent techniques"
  Under the supervision of Prof. Stergios Pirintsos

Working experience

April 2017 – to date	**Traineeship – Publishing Editorial Springer Nature** Department of Clinical Medicine

Languages

- English – Fluent
- Greek – Native speaker
- German – Fluent
- Spanish – Beginner

List of Publications

- **Glynou K**, Ali T, Buch AK, Haghi Kia S, Ploch S, Xia X, Celik A, Thines M, Maciá-Vicente JG (2016) The local environment determines the assembly of root endophytic fungi at a continental scale. Environmental Microbiology **18**: 2418–2434.
- **Glynou K**, Ali T, Haghi Kia S, Thines M, Maciá-Vicente JG. Genotypic genetic diversity in root-endophytic fungi reflects efficient dispersal and environmental adaptation. Molecular Ecology.

- Kia SH, **Glynou K**, Nau T, Thines M, Piepenbring M, Maciá-Vicente JG (2016) Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants ISMEJ doi: 10.1038/ismej.2016.140.
- Cheikh-Ali Z, **Glynou K**, Ali T, Ploch S, Thines M, Bode HB, Maciá-Vicente JG (2015) Diversity of exophillic acid derivatives in strains of an endophytic *Exophiala* sp. Phytochemistry doi: 10.1016/j.phytochem.2015.08.006.
- Maciá-Vicente JG, **Glynou K**, Piepenbring M (2016) A new species of *Exophiala* associated with roots. Mycol Progress 15:18.

Selected Conference Abstracts

- "Intraspecific genetic variability in root-endophytic fungi and implications on dispersion dynamics and environmental adaptation"
  Kyriaki Glynou, Jose G. Maciá-Vicente
  The International Conference of the German Mycological Society, Bernried, Germany, September 2016
- "Dispersion dynamics and phenotypic variability do not reflect intraspecific genetic differences in root-endophytic fungi"
  Kyriaki Glynou, Sevda Haghi Kia, Jose G. Maciá-Vicente
  The Dynamic Fungus, Exeter, UK, September 2016
- "Factors affecting the occurrence of fungal root endophytes and their interaction with plants"
  Jose G. Maciá-Vicente, Sevda Haghi Kia, **Kyriaki Glynou**
  Rhizosphere4 Congress, Maastricht, the Netherlands, July 2015
- "Geographical locality and soil physicochemistry determine the fungal endophytic diversity in roots of *Microthlaspi perfoliatum*"
  Kyriaki Glynou, Marco Thines, Tahir Ali, Jose G. Maciá-Vicente
  The International Conference of the German Mycological Society, Orscholz, Germany, September 2014
- "Finding patterns in the occurrence and plant interactions of fungal root endophytes: towards a better understanding of their ecological significance"
  Jose G. Maciá-Vicente, **Kyriaki Glynou**, Sevda Haghi Kia
  Joint Meeting of COST FA1206 (WP3) & FA 1103 Exo- & Endogenous Signalling, Kraków, Poland, September 2014
- "Diversity and biogeography of fungal endophytes in the roots of the non-mycorrhizal plant *Microthlaspi perfoliatum*"
  Kyriaki Glynou, Marco Thines, Tahir Ali, Jose G. Maciá-Vicente
  The 10th International Mycological Congress, Bangkok Thailand, August 2014
- "Genetic and chemical diversity of fungal root endophytes: insights from their ecology and interaction with plants"
  Jose G. Maciá-Vicente, **Kyriaki Glynou**, Sevda Haghi Kia, Thomas Nau
  The 10th International Mycological Congress, Bangkok, Thailand, August 2014

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- Travel Grant of the British Mycological Society for the attendance to 'The Dynamic Fungus' meeting

Additional activities and hobbies

- Speaker of the PhD students-members of the IPF Research Cluster
- Member of British Mycological Society (BMS)
- Cooking, cinema, jogging